

**ABNORMAL PLATELET REACTIVITY
AS A RISK FACTOR FOR
PREMATURE CORONARY HEART DISEASE IN MALES**

by

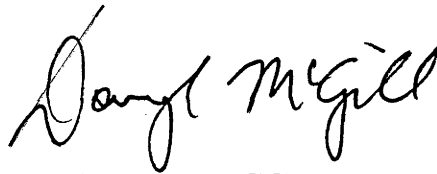
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A Thesis submitted for the degree of Doctor of Philosophy
Australian National University

May, 1993

DECLARATION

I declare that this thesis reports my own original work, except where acknowledged, and that no part of it has been previously accepted or presented for the award of any degree or diploma by any university. To the best of my knowledge, no material previously published or written by another person is included, except where due acknowledgement is given.

A handwritten signature in black ink, reading "Darryl McGill". The signature is fluid and cursive, with the first name "Darryl" and last name "McGill" clearly distinguishable.

DARRYL MCGILL

May, 1993

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I am greatly indebted to a number of people for their help, inspiration and motivation to undertake and complete the work for this Thesis. I offer them my sincere thanks, *especially* all the individuals who participated in the studies as subjects.

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children who always make it worthwhile and a beginning,
a delight.

to my parents
for their love and encouragement

QUOTATION

"Being good, conveying as it does the notion of value, implies being an end or goal, and this is the starting-point of causal action, the cause of causality itself; for no agent acts except for some goal, and no matter would acquire form unless an agent acted on itIn causal action everything starts with the good end which motivates the agent to act, and so elicit the final of the effect. In the caused thing we find the opposite order: first, the form itself brings with it existence; next, there is the operative power which brings perfection of existence (for a thing is perfect, Aristotle says, when it can reproduce itself); so that finally, the thing realises the notion of goodness. It is usual to distinguish three different meanings of good: the *useful*, the *worthy* and the *delightful*. For whatever is of value, and can satisfy desire, is good. That which satisfies as a stage on the way to something else we call *useful*; that which satisfies itself we call *worthy*, and the satisfaction found in it *delight*. So the primary sense of good is *worthy*, the second *delightful* and the third *useful*.

St Thomas Aquinas, *Summa Theologiae*

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ABSTRACT

Hypothesis: Increased platelet reactivity is a thrombogenic risk factor for the development of premature coronary heart disease (CHD), transforming coronary artery atherosclerotic disease, that is coronary artery disease (CAD), into the clinical problems of acute and chronic CHD.

Aim: To determine if males with premature CHD and angiographically proven CAD differ in platelet reactivity in comparison to a control group without CHD, and if present to attempt to determine if any difference was due to other factors, particularly CHD risk factors.

To determine if measures of platelet reactivity bear any relationship with angiographically defined CAD, whether to the extent of mural involvement or the number/severity of discrete obstructive lesions.

Method: This was a case-control study of 53 males with premature CHD and 48 age and socioeconomically matched controls without CHD. Besides specific platelet function measures, included in the comparison were the known main risk factors and a number (although not all) of more recently described and potentially important risk factors. The usual demographic information and a dietary diary were also obtained. A number of preparatory studies were also undertaken to evaluate specific potential areas of bias that may have confounded the results of the case-control study. In addition, the relationship between angiographic CAD and CHD risk factors and platelet function were evaluated

Results: As expected, the major risk factors for CHD were significantly different. Measures of platelet reactivity were also significantly different between the two groups indicating an increased platelet reactivity in the CHD group. However, no difference in measures of *in vivo* platelet activation was present, suggesting no on-going platelet activation in stable CHD patients.

Lipoproteins and fatty acid levels were independently associated with the extent of coronary artery atherosclerosis assessed angiographically, but not with the a composite score of number and severity of discrete obstructive coronary artery lesions. Increased platelet response to adrenaline correlated positively and independently with the composite severity score for the number and severity of discrete CAD lesions.

Conclusions: The results of this study, together with existing data, indicate that increased platelet reactivity is a risk factor for CHD and for the presence of severe obstructive coronary artery lesions in young males. There is substantial evidence, although not complete, that increased platelet reactivity is a causative risk factor for the development of CHD and obstructive CAD.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AMI	Acute myocardial infarction
APTT	Activated partial thromboplastin time
ATP	Adenosine triphosphate
BP	Arterial blood pressure
BTG	Beta-thromboglobulin
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CAS	Coronary atheromatous score
CHD	Coronary heart disease
CS	Collateral score
CSS	Coronary stenosis score
ECG	Electrocardiogram
GP IIb/IIIa	Glycoprotein IIb/IIIa
HbA1C	Glycosylated haemoglobin
Hb	Haemoglobin
Hc	Haematocrit
HDL	High density lipoprotein
HMF	Hydroxymethylfurfural
LAGCOL	Lag phase before shape change (collagen-induced aggregation)
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LT50	Lag time to 50% of maximal change in light transmission with platelet aggregation induced by each platelet agonist
LVS	Left ventricular score
mCAS	Mean coronary atheromatous score
mCSS	Mean coronary stenosis score
MI	Myocardial infarction
MPV	Mean platelet volume

ABBREVIATIONS CONTINUED

PCR	Platelet count ratio
PGI ₂	Prostacyclin
PF ₄	Platelet factor four
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PTCA	Percutaneous transluminal coronary angioplasty
PT	Prothrombin time
R	Maximum rate of change in light transmission with platelet aggregation induced by each platelet agonist
RIA	Radioimmunoassay
TXA ₂	Thromboxane A ₂
TXB ₂	Thromboxane B ₂
WCC	White cell count

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21 McGill D A, Ardlie N G . Variability of serum thromboxane production in males with coronary heart disease.

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CHAPTER 1

THE PATHOPHYSIOLOGY OF ACUTE CORONARY SYNDROMES, CORONARY HEART DISEASE AND ATHEROSCLEROSIS. THE ROLE OF THE PLATELET.

1.1 THROMBOSIS IN THE CORONARY CIRCULATION

1.1.1. Coronary Artery Thrombosis

The development of an occlusive thrombus superimposed on a fissured or ulcerated atherosclerotic plaque has been established as a prime component in the development of acute myocardial infarction (AMI).¹⁻⁴ This concept was expressed by James Herrick as long ago as 1912.⁵ Although the term acute myocardial infarction was originally considered synonymous with acute coronary thrombosis,^{5,6} the role of occlusive coronary thrombi in AMI had not always been fully accepted and there had been a great deal of controversy until recently.⁷ The observations that many patients with AMI did not have thrombus present,⁸ that coronary thrombosis occurred without causing AMI,⁶ and that pathological studies suggested that coronary thrombosis was a result rather than a cause of AMI,⁹ all contributed to the argument. However, recent studies using improved techniques have provided clear evidence of a role for thrombosis in the pathogenesis of AMI, and indeed, the other acute coronary syndromes.¹⁻⁴ There is a consistent relationship between the acute clinical syndromes of coronary artery disease (CAD) and the presence of arterial thrombi and/or platelet aggregates within the coronary circulation. This is particularly evident in AMI.

1.1.2. Acute Myocardial Infarction

Convincing evidence for the involvement of thrombosis in the pathogenesis of AMI has come from a number of sources. Firstly, many pathological studies have shown thrombus located in the proximal portion of a coronary artery supplying infarcted myocardium.^{6,10-13} Secondly, the delivery of intracoronary thrombolytic agents can reopen occluded arteries in the setting of AMI.¹⁴ Furthermore, with the use of radioisotopes, the central portion of a thrombus subtending an infarct has been shown to be radio-negative,¹⁵ indicating that the initial thrombus formed prior to administration of the radioisotope, and therefore early in infarct development or antecedent to infarction. The incorporation of ¹²⁵I-fibrinogen into coronary artery thrombi after the onset of typical ischaemic chest pain demonstrates that an occlusive thrombus may, however, propagate after the onset of necrosis.¹⁶

1.1.3 Unstable Angina

The mechanisms involved in unstable angina are probably similar to those occurring in AMI, although other potential causes include rapidly progressive narrowing of coronary arteries from atherosclerosis, rapid accumulation of intimal foam or smooth muscle cells, coronary artery spasm, and platelet aggregation in the presence of coronary artery spasm and/or atherosclerotic narrowing.¹⁷

Angiographic features of coronary thrombosis in patients with unstable angina have been well documented.¹⁸⁻²¹ Thrombolysis in patients with unstable angina increases the patency of arteries supplying the ischaemic area of myocardium, which suggests that thrombus, in association with atherosclerotic lesions, is involved in the pathogenesis of unstable angina.¹⁹ Lesions consistent with intraluminal thrombus have been reported in 70% of patients with unstable angina, and only in 16% of patients with stable symptoms.²⁰ The lesions frequently seen in unstable angina antemortem are

similar to postmortem angiographic lesions, namely ruptured atherosclerotic plaques with associated thrombi.²² Furthermore, progression of CAD occurs more commonly in patients with unstable angina^{21,20} and can evolve from a previously insignificant stenosis.²⁰ The common morphological features of disease progression in unstable angina are a disrupted atherosclerotic plaque and/or a partially lysed thrombus.²⁰ Clinical features of unstable angina also support a common aetiological basis with AMI. There is a significant increase in the incidence of AMI occurring shortly after the onset of unstable angina and a history of unstable symptoms prior to the onset of AMI.¹⁷ This association is suggestive of similar pathophysiological mechanisms in both conditions, a concept that has important clinical applications. The validity of these studies has found strong support from the direct observation of intraluminal coronary thrombosis by angiography in unstable angina.^{4,23}

1.1.4. Sudden Cardiac Death

Many individuals who die suddenly of ischaemic heart disease have an actively progressing thrombotic process in association with a ruptured atherosclerotic plaque.^{2,24} Electrocardiographic evidence of AMI or ischaemia has been reported in up to two-thirds of resuscitated survivors of sudden coronary death (Cobb et al 1980). Platelet aggregates have been demonstrated in intra-myocardial vessels of patients dying suddenly and unexpectedly of ischaemic heart disease.²⁵ Those dying suddenly after developing unstable angina have evidence of an ongoing thrombotic process in a major coronary artery.²⁶ In addition, many patients with unstable angina and sudden cardiac death have platelet aggregates confined to vessels immediately downstream of a major epicardial coronary artery containing thrombus on a disrupted atherosclerotic plaque.²⁷ Coronary thrombosis is found in most deaths caused by AMI and platelet/fibrin microemboli have been frequently observed.¹⁵ Although the terminal event in sudden cardiac death is usually some form of ventricular electrical instability, it appears that in many sudden cardiac deaths, the pathologic process may include the rapid formation of a plaque fissure, or rupture, with subsequent thrombus formation and myocardial ischaemia. The first manifestation of this process may be a fatal ventricular arrhythmia. Nevertheless, coronary artery thrombosis does not occur in all cases of sudden cardiac death,^{28,29} and, indeed, has been reported in as few as 19% of sudden deaths.^{30,31}

1.1.5 Plaque Fissuring and Progression of CAD

Disruption of small atherosclerotic plaques with ensuing mural thrombosis and fibrotic organisation of the thrombus may contribute to the progression of atherosclerosis. Exactly how prevalent this process is, and of what clinical significance, remains unknown.³² Plaque fissuring is most frequently associated with an eccentric lipid pool in the arterial intima, and the lateral margin of plaques is the most common site of intimal tearing.³³ The site of plaque fissuring is influenced by variation in the mechanical strength of the cap of the plaque tissue due to focal foam cell accumulation.³³ Alternative, or co-existing mechanisms also probably contribute, since intimal tears can occur where there is no lipid pool within the intima.³³ Other factors appear to be involved in the progression of CAD since the extent of atherosclerosis only in part determines the prognosis of clinical coronary heart disease (CHD). Coronary thrombosis and CHD may not be the simple and direct result of the presence of

atheroma alone,^{11,34-37} and CHD is not a simple, proportional function of coronary atherosclerosis.³⁷ Some individuals with severe atherosclerosis do not experience occlusive episodes, and others with less severe disease develop major clinical manifestations.^{38,39}

1.1.6 Determinants of Plaque Fissuring and Thrombosis

1.1.6.1 Local factors related to atherosclerotic lesions. The degree and depth of plaque disruption is a prime determinant of the extent of intra-luminal thrombosis subsequently occurring.^{40,41} In addition, the degree of residual arterial stenosis can cause increased platelet deposition, due to increased shear stress induced platelet aggregation.⁴² This observation is indicative of the increased thrombogenic potential of more severe stenoses that have been observed in clinical studies.⁴³

1.1.6.2 Local factors causing platelet activation. Localised platelet activation and platelet secretion at the site of plaque rupture potentiates thrombus formation.⁴⁴⁻⁴⁶ Moreover, residual thrombus has a strongly thrombogenic surface⁴⁷ and disruption of the thrombus by intrinsic fibrinolytic mechanisms may increase platelet activation, which also occurs with pharmacologically induced thrombolysis.⁴⁸⁻⁵⁰

1.1.6.3 Vasoconstriction. Platelet and thrombin dependent transient vasoconstriction accompanying plaque disruption may further increase shear stress induced platelet aggregation, and further aggravate the potential for ongoing thrombosis.^{41,51}

1.1.6.4 Systemic thrombogenic factors. The search for a systemic thrombogenic or hypercoagulable state has been a long-term quest⁵²⁻⁵⁵ and continues to attract considerable attention.^{41,56} Known potential factors include increased platelet sensitivity,⁵⁷⁻⁵⁹ increased circulating fibrinogen levels⁶⁰⁻⁶² and factor VII levels,⁶⁰ defective fibrinolysis⁵⁶ and increased blood viscosity.^{63,64}

1.1.6.5 Metabolic and hormonal influences on thrombosis. The effects of abnormal lipoprotein levels and plasma fatty acids on thrombosis and platelet function are potentially detrimental.^{53,65-67} Enhanced platelet reactivity at the site of vascular damage in experimental hypercholesterolaemia has been well documented.⁶⁸ Lipoprotein(a), a molecule similar to low density lipoprotein (LDL), has a glycoprotein, apolipoprotein(a), which has close structural homology with plasminogen.⁶⁹ This lipoprotein when occurring in increased amounts is a risk factor for CHD, possibly as a thrombogenic risk factor with a causative association with CHD events.⁶⁹ The potential for increased secretion of adrenaline in patients with CHD, and in association with other risk factors,⁴¹ has important implications since this hormone contributes to platelet aggregation.⁷⁰⁻⁷² Many other promising metabolic abnormalities, such as increased homocysteine levels, may provide further insight into the thrombotic component of CHD.⁷³

1.2 PLATELET FUNCTION IN ACUTE CORONARY SYNDROMES

Platelet activation appears to play a pivotal role in the development of the acute obstructive ischaemic syndromes of CHD. The evidence for this comes from a number

of sources indicating that platelet activation is important in the pathophysiological process of acute coronary thrombosis. *It remains unclear, however, whether preexisting platelet activation predisposes to thrombus formation.*

1.2.1 Acute Myocardial Infarction and Platelets

Platelet activation has been demonstrated in patients with unstable angina and early during AMI.^{45,74} Changes in some measures of platelet function indicating platelet activation are well documented. Firstly, an elevation of beta-thromboglobulin (BTG) early during AMI has been reported.⁷⁵⁻⁸¹ Increased BTG levels has been reported to be directly proportional to the severity of CAD.⁷⁷ Other studies have found increased levels of BTG in patients with CAD which does not increase further with AMI,⁸² while others have shown increases in BTG only in a minority of patients with AMI.⁸³ Secondly, most reports have also found an elevated platelet factor 4 (PF4) in the early phases of AMI.⁸⁴⁻⁸⁸ However, others have failed to demonstrate an increase in PF4 except in association with invasive procedures or platelet degranulation in vitro.⁸⁹ Another study reported an increase in PF4 in stable chronic CHD and no further increase associated with AMI.⁸⁷ Thirdly, a metabolic product of thromboxane A2 (TXA2) excreted in the urine would appear to be a reliable measure of platelet activation since it is not associated with artefactual changes due to sampling procedures.⁴⁵ The metabolic product, 2,3-dinor-thromboxane B2 is clearly elevated in the urine of patients with AMI and unstable angina.⁴⁵⁻⁹⁰ Increased TXA2 formation must therefore occur at least in the early phase of AMI and unstable angina. Whether there is a pre-morbid increase contributing to the initiation of the events is uncertain. Interestingly, platelet hyper-reactivity may be related to prognosis in AMI⁵⁹ and CHD.⁹¹ Finally, activation of prostacyclin (PGI2) synthesis occurs in these conditions,^{45,90,92} although there may not be an increase in PGI2 production in many patients during the acute thrombotic phase of AMI.⁹³

Measures of platelet survival time do not appear to be strongly influenced by acute ischaemic syndromes of CHD. Platelet survival time is shortened in chronic CHD,⁹⁴⁻⁹⁶ although during AMI there appears to be no additional measurable reduction in platelet mean lifespan above that observed in chronic CAD.⁹⁶

Clinical studies with aspirin support the evidence for platelet involvement in these acute occlusive coronary artery syndromes of unstable angina and AMI.⁹⁷⁻¹⁰³ Although the mechanism(s) for the beneficial influence of aspirin has not have been fully delineated, it is assumed to be the inhibitory effect on platelet cyclooxygenase, thereby inhibiting platelet aggregation. Aspirin has also been effective in secondary reduction of coronary occlusive events after AMI,^{104,105} probably by preventing rethrombosis.

1.2.2 Unstable Angina and Platelets

The clinical syndrome of unstable angina encompasses a diverse spectrum of patients and clinical presentations. Within those groups diagnosed as having unstable angina pectoris, there are certain subgroups with an increased risk of early myocardial infarction (MI) and even death.^{17,106-109} Angiographic,^{18-20,26,110-115} autopsy,^{26,112} as well as angioscopic^{4,23} evidence indicates that thrombus formation over a fissured

atherosclerotic plaque plays a key role in the development of unstable angina. Not surprisingly then, platelet activation has been demonstrated in unstable angina reflected by elevated plasma levels of platelet derived proteins, BTG and PF4^{74,76,81,83,116} Furthermore, increased production of TXA2 as measured by urinary metabolites is evident.^{45,74} In unstable angina, there is a higher thromboxane B2 (TXB2) metabolite excretion in patients with persistent ischaemic symptoms than in those that respond to treatment. Similarly, higher levels are found in those with post infarctional angina.⁷⁴ Enhanced excretion of TXB2 metabolite is frequently associated with angiographic evidence of thrombosis.⁷⁴ In addition, prospective clinical trials have shown a reduction in CHD events and in mortality with antiplatelet drugs.⁹⁷⁻⁹⁸ Heparin also decreases the incidence of AMI associated with unstable angina.^{103,117}

1.3 PLATELETS, PLATELET FUNCTION AND CORONARY HEART DISEASE RISK FACTORS

The major risk factors associated with CHD may also independently contribute to increased platelet reactivity in individuals with CHD. The clinical, pathological or epidemiological relevance of this to the development of CAD and CHD remains clouded. The concept of risk factors for CHD is discussed fully in section 1.4.

1.3.1 Platelets and Tobacco Use

Cigarette smoking is a major risk factor associated with the development of clinical CHD. The frequency of various forms of arterial thrombosis, the overall mortality from CAD and that of sudden death alone, all increase with the number of cigarettes smoked by the individual.¹¹⁸⁻¹²⁰ The mechanism(s) by which smoking increases the likelihood of developing CAD are unknown, although various factors, including platelet dysfunction, have been incriminated. Chronic smokers have shortened platelet survival and increased platelet turnover.¹²¹ However, platelet survival time is lengthened in smokers, with or without CAD, who abstain from smoking.⁵⁷ An acute effect of smoking may be to increase platelet aggregability,¹²² independently of the nicotine concentration.¹²³

1.3.2 Platelets and Dyslipoproteinaemia

Various forms of hyperlipoproteinaemia appear to induce changes in platelet composition and function. However, both the effects of hyperlipoproteinaemia on platelet composition, reactivity and function, and the contribution of any such effects to atherogenesis are poorly understood and controversial. Hypercholesterolaemia (Fredrickson's types IIa and IIb) may be associated with increased platelet sensitivity to aggregating agents,¹²⁴ and a reduction in platelet survival time,¹²⁵ although this is contentious.¹²⁶ An increased sensitivity to aggregation appears to be associated with an increased cholesterol content of the platelet membrane.¹²⁷ In vitro cholesterol loading of platelets results in a marked augmentation of TXA2 production in the presence of thrombin,¹²⁸ a finding consistent with the observation of an increased production of TXA2 in type IIa hyperlipoproteinaemia.¹²⁴ Moreover, a low fat diet has been reported to decrease the aggregability of platelets,¹²⁹ and a large meal of saturated fats causes increased platelet reactivity.¹³⁰ If there are significant changes

in platelet function in association with the hyperlipidaemias occurring in those susceptible to CHD, such changes may be detectable before atherosclerosis becomes advanced. These aspects are discussed more fully in Chapter 5.

1.3.3 Modifications of Low-Density Lipoproteins

Characteristics of low-density lipoprotein (LDL) particles, other than the plasma concentration, can influence platelet behaviour.^{131,132} Oxidative modification of LDL has gained considerable attention because of the substantial evidence indicating that the process occurs *in vivo*, and the compelling evidence for its role in atherogenesis.¹³³ Oxidised LDL has numerous biological properties,¹³¹ including an ability to interact with platelets and increase their aggregability.¹³⁴ Also, in theory, a deficiency of antioxidants within the body may reduce PGI₂ production, and hence promote platelet aggregability.¹³² However, the effects on platelet aggregation of antioxidant supplementation remain inconsistent.^{132,135}

Other types of LDL modification, such as the non-enzymatic glycosylation of LDL,^{136,137} may also be important in the atherogenic process.¹³³ Moreover, platelet aggregation is increased in the presence of glycated LDL.¹³⁷

1.3.4 Blood Glucose

The adverse effects of abnormal glucose metabolism on platelets has been extensively investigated and previously reviewed.¹³⁸ For example, elevated blood glucose influences adenosine diphosphate (ADP)-induced platelet aggregation,¹³⁹ thromboxane and PGI₂ biosynthesis,^{140,141} and raised plasma adrenaline and hyperglycaemia after MI may activate platelets which could contribute to poor outcome in such patients.⁷⁵ Whether such influences on platelet function account for or contribute to the increased risk in diabetics for the development of clinical syndromes related to atherosclerosis remains uncertain. Other potential sources for increasing risk include abnormal lipoprotein metabolism¹⁴² and glycosylation of proteins such as the protein in LDL, thereby possibly making the molecule atherogenic.^{133,136,137} Moreover, fibrinogen is elevated in diabetics^{143,144} and there are potential adverse effects on coagulation and fibrinolysis.¹⁴⁴

1.3.5 Hypertension and Platelet Function

A relationship between blood pressure (BP) and platelet aggregation appears to exist.¹⁴⁵⁻¹⁴⁷ For example, patients with a mean arterial BP greater than 120 mmHg have an increased tendency for platelet aggregation to ADP and adrenalin, which is normalised when the mean arterial pressure is reduced by treatment.¹⁴⁶ Although the relationship between the systolic or diastolic BP of individuals or groups and platelet function has not been extensively evaluated, the concept is reviewed elsewhere.¹⁴⁷

1.4 CORONARY HEART DISEASE RISK FACTORS

A risk factor is a statistical predictor of disease and its identification is usually based on associations demonstrated between the predictor and the disease in epidemiological studies. As a consequence, a risk factor may or may not be causally related to

the disease. For example, it may be a secondary manifestation of a more basic underlying abnormality or an early symptom of the disease.

1.4.1 Epidemiology

The established "causative" risk factors of CHD fail to account for the total incidence of CHD.^{39,148-158} Nor do they explain the absence of clinical CHD in persons with these risk factors.¹⁴⁸ Furthermore, the known risk factors have a limited ability to predict disease in an individual,¹⁵² and this low predictive accuracy limits their use for identifying persons with asymptomatic CAD. Indeed, the low predictive accuracy for identify individuals with CHD also applies to groups.^{153,159} The results of the Pooling Project defined a wide range of factors increasing the risk for CHD within different populations, and demonstrated significantly increased risk with levels of associated factors well below the 95th percentile, particularly when occurring in combination.¹⁴⁸ These observations have profound implications for public health and the prevention of premature CHD. For the individual, these results have illustrated major limitations in the ability to prospectively predict disease occurrence, even when the strongest risk factors are combined. Quantification of risk factors in the form of a decile risk has been used to determine the likelihood of developing CHD.¹⁶⁰ In those with the lowest risk it was 5%, and in those with the highest risk it was 21.4% over 8 years.¹⁶⁰ This synergistic effect of the major risk factors also remains to be explained. Use of risk factor profiles can identify 10% of the general population who will have 22% of the CHD in the population.¹⁶⁰

A number of observations made in epidemiological studies further illustrate the incomplete foundation that known major risk factors make for explaining all CHD. Firstly, international population studies reveal a significant variation in CHD mortality in different countries.¹⁶¹ This variation is probably only partly due to population differences in blood cholesterol levels.¹⁶² It should be emphasised that cross-cultural comparisons do indicate that CAD is rare in populations with cholesterol levels less than 4.2 mmol/l.¹⁶² However, regional differences within some countries cannot be wholly explained on the basis of differences in levels of the 3 major risk factors for CHD. Secondly, the Framingham Study logistic, useful for predicting CHD in high risk population groups, over-estimates CHD incidence in "low risk countries". This indicates that the influence of risk factors in the predominantly Caucasian population of Framingham is greater than in non-Westernised countries. The reason for this observation is unknown, but may simply be due to the lack of underlying atherosclerosis in populations with low cholesterol levels leaving no substrate for the other "causative" risk factors to act upon and result in obstructive lesions causing clinical CAD. Thirdly, even though total cholesterol levels predict long term CHD mortality it is not a major factor in predicting all-cause mortality.¹⁵⁹ The association between cholesterol and CHD at "borderline hyperlipidaemia" may reflect the correlation of cholesterol with other risk factors.¹⁵⁹ Finally, and of particular relevance for the hypothesis in this Thesis, the incidence of CHD in France is relatively low compared to other Western Countries with equal intake of saturated fats and population cholesterol levels.¹⁶³ Moreover, other risk factors for CHD, including BP levels, body-mass index, and cigarette use for men are no lower in France than in other industrialised countries.^{163,164} One proposed explanation for this variation is the inhibition of platelet reactivity by alcohol.¹⁶⁴

Even though population studies have shown that all of the variance for CHD development in a population cannot be explained by the major risk factors,¹⁴⁸ there are caveats to this observation. A limited number of risk factors have been included in these studies. There may also be the effects of longitudinal trends on the variables investigated, either increasing if favourable, or decreasing if unfavourable. Further, the majority of persons who have a MI are in the second, third and fourth quintiles for cholesterol level, as are the majority of persons who do not have CHD.^{165,166}

1.4.2 Risk Factors and Coronary Artery Pathology

The strength of association between CHD risk factors and pathologically defined atherosclerosis also has limitations. The observation that atherosclerosis is closely associated with major risk factors is only true if the correlation is estimated on a group basis. The correlation is much weaker when estimated on an individual basis.¹⁵² For instance, correlation coefficients between cholesterol level prior to death and quantitatively evaluated coronary atherosclerosis range from 0.15 to 0.35 for the individual analysis, but 0.76 for groups. This indicates that individuals with higher cholesterol levels are not all equally susceptible to CHD.¹⁵² Furthermore, there is a progressive rise in the prevalence of all major risk factors with age, in parallel with a continual reduction in the predictive accuracy.^{151,167} When comparing homogeneous age-sex-race groups without significant risk factors, the range of the extent of CAD in any single arterial segment is similar between groups, but there is up to a 5 fold difference in the mean measure for the extent of disease for all the coronary arterial tree between the groups.¹⁵³ That is, the median varies between groups. Variability among arteries in the same individual implies that the response of the arterial wall differs, assuming that the composition of the blood reaching each artery is identical. Indeed, there is a wide variation in the extent of raised lesions in male smokers and non-smokers with an enormous overlap despite significant differences in mean values.¹⁵⁸ Even though the presence of CAD in a population correlates with the incidence of CHD, and CHD cases almost always have more atherosclerosis on the average than non-CHD cases in the same location, the incidence of CAD is significantly greater than the incidence of CHD, implicating other factors as well as the presence of atherosclerosis in the development of CHD.³⁹ Furthermore, even after classifying cases according to risk factors in a prospective autopsy study, there can be individual variation in the extent of raised atherosclerotic lesions, and the risk factors may only explain about 26% of the individual variation in raised lesions.¹⁶⁸

Important determinants of whether an individual develops CHD may be local factors within the artery and/or may be related to the morphology of the atherosclerotic plaque. Indeed, mechanical, hydraulic and cellular/molecular characteristics of the plaque may be important determinants in the development of clinically significant lesions.¹⁶⁹ For instance, although the distribution of circumferential tensile stress across the intima is altered by the atherosclerotic plaque, and areas of high stress correlate with the site of intimal tears,³³ these sites are also affected by variation in the mechanical strength of the intimal cap.³³ Sites of intimal tears appear to be weak points which are largely determined by focal accumulation of foam cells.³³

1.4.3 Risk Factors and Angiographic CAD

Although risk factors are clearly associated with both clinical manifestations of CAD and angiographically demonstrated CAD, they are not accurate predictors of angiographic CAD.¹⁴⁹ Between 9% and 17% of male subjects have no definable risk factor for CHD in the presence of angiographically defined CAD.¹⁴⁹ In addition, limited gains accrue from the use of discriminant function analysis in correctly allocating patients into angiographically defined disease and non-disease groups according to the major risk factors.¹⁴⁹ This topic is elaborated on in Chapter 8.

1.4.4 Genetic and Hereditary Factors

Family studies consistently demonstrate a prominent familial grouping of CHD. In males, there is likely a genetic predisposition to both premature CHD,^{154,155,170-175} and the major risk factors for CHD.^{154,155,165,173,176} The familial occurrence of CAD is well documented by case-control,¹⁷⁷⁻¹⁸⁰ prospective,¹⁸¹ and twin studies.^{182,183} No discrete genetic mode of inheritance has emerged for the majority of family aggregates with CAD and only occasionally have single gene disorders accounted for the development of premature CAD in a general population.¹⁸⁴ A family history of MI is an independent risk factor for MI, and the number of relatives and the age at which they are affected is related to the strength of the association.¹⁸⁰

The tendency for first degree relatives of CAD patients to have more adverse risk factors than the general population has been well documented,^{176,178,185} particularly so when CAD is manifest at a young age.¹⁷⁶ The major risk factors per se have a substantial familial component, whether of genetic or environmental origin. It may be that the contribution of genetic factors is greater than can be attributed to the inheritance of the major risk factors alone.¹⁸⁶ For most of the important risk factors, familial associations arise from both a shared genotype and a shared environment.¹⁸⁷

The familial nature of CAD may be explained in part by common environmental factors shared by family members. Nonetheless, the classic risk factors do not entirely predict familial occurrence of CHD, even if the genetic risk factors are eliminated.^{165,173} It is even possible that the risk ratio for a positive family history of CHD is greater than for the highest quintile of cholesterol levels,¹⁶⁵ and that the contribution of heredity may exceed that of environment.¹⁷³

The exact nature of the genetic factors, which are independent of the known risk factors and which impart a predisposition to CAD, are obscure. In addition, for young males, the heredity association is particularly important,¹⁷⁰ and it may even be restricted to younger men.¹⁸⁸ The relationship in females is less certain.^{175,188} Unfortunately specific inheritance patterns, genetic mechanisms or gene-environment interactions for CAD have not been well defined and require more intense evaluation.^{154,155,170,173} Diseases attributable to multifactorial inheritance are caused by the small effects of many genes, and only in a small number of cases are determined by single genes of large effect.^{189,190}

It is assumed that there are multiple predisposing hereditary factors that interact with environmental triggers to produce CAD, rather than a single gene mode of inherit-

ance. Evidence exists, however, that in patients susceptible to CHD there are single major gene influences, and, although research has concentrated on lipid disorders, there may be major gene influences other than through lipoprotein abnormalities.¹⁶⁵ For instance, 'abnormal' platelet response to ADP have been found more often in the close relatives of young men with AMI than in a general population.¹⁹¹ Indeed, other heritable risk factors appear to contribute strongly to the occurrence of premature CAD.¹⁹²

Risk factors other than those currently known must mediate the effects of the family aggregation of CAD.¹⁹³ The presence of an environmentally sensitive inherited abnormality of platelet reactivity is one possibility that requires evaluation.

1.4.5 Sex Differences in CHD

Differences in known risk factors fail to explain the male sex predominance in either atherosclerosis or CHD incidence.^{152,155,194-196} However, there is an absence of detailed studies of the variability in CHD susceptibility between men and women.¹⁹⁶ There are also unexplained differences in the prognosis related to CAD risk factors¹⁹⁷ and the presence of CHD.^{194,195,197} For example, women appear to have much lower rates of CHD than men at the same values of cholesterol and increased risk at higher levels of cholesterol.¹⁹⁸ Furthermore, there are important differences in the natural history,¹⁹⁵ the risk factor profile^{194,195,198,197} and pathology^{199,200} of CHD in women compared to males. However, most young women with overt CHD have a detectable risk factor, but also have a high rate of non-atherosclerotic MI.²⁰⁰

1.4.6 Conclusion

Despite the concerns discussed above, epidemiological evidence is persuasive that the 3 major risk factors (tobacco use, hypertension and hypercholesterolaemia) are causative in their association with CHD,¹⁶¹ in particular, lipid abnormalities.²⁰¹ Even though a calculated attributable risk for these factors may be as high as 90%,²⁰² their lack of diagnostic specificity and low predictive accuracy for the development of CHD remains to be explained. Many individuals remain free of the clinical manifestations of CAD even when multiple risk factors are present.^{148,160,166,203,204} Furthermore, their poor predictive accuracy persists into old age^{151,160,167,205} and, while certain factors are significantly correlated with angiographic findings, their value for predicting the severity of CAD disease is also very limited.^{149,206,207} Indeed, in the younger patient the progression of coronary atherosclerosis into distinct coronary artery stenosis may largely be determined by mechanisms other than the presently known risk factors.²⁰⁷ Based on present evidence, there seems to be at least two processes involved in the development of atherosclerotic vascular disease, one in response to localised lipid accumulation within the vessel wall and the other a thrombotic response leading to more rapid development of stenotic coronary artery lesions.⁹

1.5 THE CONCEPT OF THROMBOGENIC RISK FACTORS

In this section it will be argued that, beside the pivotal role that coronary thrombosis

has in the pathogenesis of the acute ischaemic syndromes of CHD, a predisposition to arterial thrombosis may exist as a risk factor for the different stages leading to the clinical expression of CHD. That is, thrombogenic risk factors may be important in the pathogenesis of atherosclerotic plaque formation as well as obstructive thrombosis.

1.5.1 Coronary Thrombosis

As discussed above, thrombus superimposed on a fissured atherosclerotic plaque is usually present in the coronary artery supplying infarcted myocardium,^{1,3,13} and other acute coronary artery syndromes.²⁰⁸⁻²¹⁰

1.5.2 Progression of CAD

The formation of severe atherosclerotic lesions may develop following recurrent episodes of localised thrombus formation,^{10,11} with concomitant fragmentation of thrombus.¹¹⁵ Support for this concept arises from a number of pathological studies. Microemboli and/or microinfarcts found in the myocardium distal to evolving coronary thrombi, indicate the dynamic aspect of intravascular thrombosis.^{3,25,211} Fibrin appears to be a major component of atherosclerotic lesions,²¹² which is compatible with the incorporation of fibrin into the lesion following recurrent mural thrombosis. Alternatively, this fibrin may be formed from fibrinogen found within the intima.³⁴ In addition, intimal haemorrhage, a well known complication of atherosclerosis,²¹³⁻²¹⁵ may cause rapid progression of atherosclerotic lesions. These haemorrhages may be due to intimal dissection from the lumen,^{2,11,216} or arise as a sequelae to neo-vascularisation of the intima.^{213,214,217,218}

In an autopsy study of the evolution of atherosclerotic lesions in young individuals, fibrous caps were found to form slowly.⁹ When platelets and fibrin accumulated on the endothelial surface, the caps thickened and reduced the arterial lumen quickly. The deposition of platelets and fibrin on the surface of atherosclerotic lesions was thought to be the cause of the collagenous caps and the main cause of the rapid progression to lumen stenosis. This was found to be an uncommon process up to the third decade when most caps were thinner with little collagenous thickening when compared with cases having thrombotic deposits.⁹ The latter were observed in the third decade of life in some subjects and were episodic in their occurrence.⁹ This autopsy study provides evidence of lesion progression consistent with angiographic studies showing rapid lesion development in areas of minimal disease. On the other hand, lipid cores often begin to develop soon after puberty and increase linearly over time.⁹ Proliferation of smooth muscle cells and synthesis of collagen forming the cap develops after a lipid core evolves.⁹

1.5.3 Extent and Severity of CAD

The concept that coronary thrombosis and CHD may not be the simple and direct result of the presence of atherosclerosis alone, has been previously considered on the basis of results from historical and epidemiological necropsy studies.^{11,34-37} The suggestion that "ischaemic heart disease is not a simple, proportional function of coronary atherosclerosis"³⁷ is an important concept in understanding the aetiology of

CHD. There are a significant number of individuals who have AMI and are subsequently shown to have angiographically near normal, or normal, coronary arteries, particularly younger patients.²¹⁹⁻²²² Younger patients also have less extensive coronary atheroma.²⁰⁷ Furthermore, in necropsy studies, substantial differences in the frequency of myocardial fibrosis have been observed which correspond only moderately to the frequency of coronary occlusions, and which do not relate at all to the frequency of lesser narrowing of the coronary lumen.³⁵ If plaque fissuring, subsequent coronary thrombosis, and hence coronary occlusion were related to the arterial burden of atherosclerosis alone, it would be reasonable to assume some quantitative association between the extent of myocardial fibrosis due to MIs and coronary atherosclerosis. When the prevalence of coronary atherosclerosis recorded in postmortems in the United Kingdom for the years 1908-13 was compared with that for the years 1944-49, there appeared to be some reduction in the amount of mural atherosclerosis in the population, while at the same time there was a tenfold increase in deaths from ischaemic heart disease.³⁷ Obviously there must be other factors operating to produce CHD, other than simply the presence of atherosclerosis, albeit an important prerequisite.

1.5.4 Circadian Variation of Coagulation and Myocardial Ischemia

Evidence now exists that AMI is not entirely or necessarily a random event occurring at any time of the day. The circadian variation in the occurrence of AMI is well documented,^{223,224} the peak onset being between 6.00am and 12.00am.²²³ The authors of these publications have documented an extensive number of older studies from throughout the world showing a remarkable similarity in the time of onset of rest angina and AMI. In addition to the circadian variation in clinically apparent and also asymptomatic CHD, there is increasing support for the presence of a circadian rhythm of some of the components of thrombus formation, predisposing to a hypercoagulable state during the peak period for the onset of AMI. Numerous changes occur during this period. A decrease in fibrinolytic activity of blood,²²⁵ increased *in vitro* platelet aggregation,²²⁶ increased *in vivo* platelet activation in patients with atherosclerotic vascular disease,²²⁷ increased metabolism of pharmacological doses of heparin,²²⁸ and also increased blood fibrinogen levels in normal subjects²²⁶ have all been demonstrated. Thus, an increase in coagulability may be sufficient to coincidentally sustain thrombus formation on the random occurrence of plaque rupture. Alternatively, an environmental and/or heritable factor may be required to create a sufficiently hypercoagulable state.

1.5.5 Age and CAD

The distribution of coronary atherosclerosis in the elderly is similar to that in younger age groups,²²⁹ although there are differences in the pathological composition.^{230,231} Increased calcification and fibrosis, and atrophy of the media occur with aging.²²⁹ The incidence of CAD increases from approximately 46% in the 6th decade, to 84% in the 9th decade.²³² Not surprisingly, the Framingham Study demonstrated a continuing increase in the frequency of the clinical events of CHD with age in women. However, in men the incidence of clinical events declined in the 7th and 8th decades, but subsequently rose in the 9th and 10th decades.²⁰⁵ The increased risk of CHD in males occurred in the 6th decade in the Framingham

Study, but in the 7th decade in eastern Finland.²³³ If the acute occlusive syndromes of clinical CAD were a random event related to the chance occurrence of plaque rupture, then there should be a progressive, unabated rise in the incidence of CHD with age since the occurrence of coronary atherosclerosis increases with age. The prevalence, however, may decline due to attrition from premature deaths.

In the very elderly, the incidence of clinical CAD is approximately equal in men and women.^{205,234} Indeed, age is the strongest cardiovascular risk factor.^{202,235} Furthermore, in patients who die suddenly of ischaemic heart disease, coronary thrombosis in the presence of significant CAD was found more frequently in a younger age group, despite a tendency for the older group to have more extensive disease.³⁶ Although, more than 60% of sudden deaths in the elderly are associated with severe CAD,²²⁹ thrombus formation may play less of a role in the older patient with CHD manifestations.³⁶ Nevertheless, the relative contributions of stenosis, thrombosis and arterial spasm in the development of acute ischaemic syndromes in the elderly are unknown.²²⁹

1.6 ATHEROGENESIS

Old theories about the pathogenesis of atherosclerosis, although not as sophisticated as their modern equivalents, do resemble in broad principle current perceptions. One of the earliest documented postulates was the 'insudation' theory of Virchow (1821-1902). He proposed that atherosclerosis resulted from a chronic inflammation of the intima with intimal damage being a very early step in the process. In his hypothesis, a principal factor in progression of plaque formation was an increase in passage into and accumulation of plasma constituents in the intima, although this occurred late in the sequence.²³⁶ Although Virchow's fame relates to his contribution to the understanding of thrombosis and embolism, he did not develop an active interest in coronary thrombosis.²³⁶

In contrast, the 'encrustation' theory of Rokitansky (1804-1878) postulated that small mural thrombi on areas of arterial wall intimal injury become organised and invaded by smooth muscle cells with gradual subsequent growth. Rokitansky is quoted as saying that "the deposit is an endogenous product derived from blood, and for the most part from fibrin".²³⁶ His theory attributes more weight to environmental factors causing endothelial injury and the triggering of arterial thrombus. The lipid occurring in the plaque was felt to be a passive phenomena and a secondary product of a 'sponge-like' action of thrombus and breakdown of platelets and white cells. Baron Karl von Rokitansky felt that chronic inflammation as proposed by Rudolph Virchow was not an important feature in the development of atherosclerosis.²³⁶

Current evidence supports the probable occurrence of both processes in the pathogenesis of atherosclerosis. At present all theories need to embrace the fact that atherosclerosis development begins early in life and is a process continuing over many years.^{7,9,237} In addition, manifestation in the form of the clinical syndromes may be a further step in the development of atherosclerosis resulting from superimposed thrombosis on ruptured lesions.^{2,4,115,238}

1.6.1 Response to Injury Hypothesis

Normally, endothelial cells form a continuous non-reactive and non-thrombotic layer which also mediates metabolic exchange through the arterial wall.²³⁹ It is a permeability barrier controlling the passage of small and large molecules from plasma into the arterial wall. The endothelial surface is polarised and the luminal surface is non-reactive. Synthesis and secretion of connective tissue proteins is a major function of the endothelium. Types III and IV collagen are produced beneath the luminal surfaces and a myriad of glycoproteins and proteoglycans are secreted from the luminal surface. These latter factors contribute to the thrombo-resistant activity of the endothelial surfaces.

The response to injury hypothesis postulates that injury occurs specifically to the lining of the endothelium of the artery.²⁴⁰⁻²⁴² This injury may be non-specific, of various forms, intermittent or continuous, and acute or chronic. If the injury is limited, the process may be reversible and the integrity of the arterial wall reconstituted. If the injury is severe, continuous and/or repeated, then progression may occur due to continuing interaction of the components derived from the blood at sites where lesions were already formed. Injury may modify the equilibrium of the endothelium between cell proliferation and cell destruction. This modification would determine if a lesion progressed, remained constant or regressed.^{242,243} The endothelial injury may be of very diverse forms,²⁴⁴ including mechanical,^{245,246} injury from chemical compounds such as the lipoproteins,^{241,247} toxins,²⁴⁸ immunological insults,²⁴⁹ and even infective agents such as viruses.²⁵⁰ These diverse range of agents causing endothelial injury may lead to separation between endothelial cells with subsequent altered endothelial permeability and even frank desquamation of cells.

The major elements in this scheme are cells within the blood, namely monocytes and platelets, and cells in the arterial wall, such as the endothelial and smooth muscle cells. Sufficiently severe injury would result in the adhesion of platelets and monocytes to the endothelial wall. Thereafter, monocytes may penetrate the intima and also platelets may aggregate leading to mural thrombus formation. During this process, activation of platelets and monocytes leads to the release of substances which further interact with the arterial wall cells. These may in fact stimulate migration of smooth muscle cells from the media into the intima and induce proliferation of smooth muscle cell growth.²⁵¹ It has also been noted that the most severe atherosclerosis in rabbits occurs in areas where endothelial cells had grown over previously denuded areas but not in areas denuded and covered by platelets.²⁵²

Abnormalities of lipid and lipoproteins can also be incorporated into this hypothesis. An increase in binding of lipoprotein lipid by proteoglycans associated with newly covered endothelial areas²⁵³ appears to increase cholesterol synthesis and decrease hydrolysis of cholesterol.²⁵⁴ Lipoproteins have been shown to injure arterial endothelium and produce increased permeability to macromolecules.^{244,255} There is also evidence that monocytes can penetrate the intact endothelium in the presence of excessive amounts of LDL cholesterol in the blood resulting in changes similar to that following loss of endothelial integrity.²⁴² Indeed, the probable interaction of arterial injury, lipoprotein insudation, and platelet interaction contributes to the pathogenesis of atherosclerosis.^{244,255}

1.6.2 The "Cholesterol" Hypothesis and Oxidatively Modified LDL

The nature of the relationship between cholesterol and atherosclerosis has been debated for most of this century. Abnormal lipoprotein metabolism, of which an elevated cholesterol is but one measurable mode of expression, clearly plays an important part in the development of atherosclerosis.^{133,256-260} What exactly should be done for an individual from the general population with a moderately elevated cholesterol, however, is still debated.²⁶¹⁻²⁶⁵ The results of some studies,²⁶⁶ and potential adverse problems,²⁶⁴ still provide concern for population based primary prevention. A therapeutically positive approach is recommended for the treatment of an elevated cholesterol for the purposes of secondary prevention.²⁶⁷⁻²⁷⁵

Over many years, research into atherosclerosis concentrated on the role of circulating lipoproteins, providing a cogent body of evidence for their role in atherogenesis.²⁵⁶ More recently, as seen in the foregoing discussion, attention has also focused on cellular and molecular abnormalities in the arterial wall and circulation.^{242,276,277} Indeed, more recent data provides support for proposals interrelating the cellular process and circulating lipoproteins.^{131,133,259,260}

The evidence that oxidative modification of LDL may play a role in atherosclerosis has been increasing rapidly over the last few years.^{133,278} Oxidative modification of lipids, that is lipid peroxidation, is a free radical mediated process that involves the peroxidative degradation of lipid material. In its broadest sense, lipid peroxidation includes the peroxidase reactions of all types of lipid materials, including free fatty acids, phospholipids, triglycerides, and cholesterol. Particular attention has focused on the peroxidation of LDL.²⁶⁰ This process forms an important part of the oxidative modification of LDL hypothesis for atherogenesis which has become a well established concept.²⁶⁰ Such oxidative modification of LDL allows the uptake of lipids by intimal monocyte derived macrophages and accumulation of lipids, in various forms, in the wall of the artery.^{131,133,259,260} Indeed, the cytotoxicity and chemotactic effects of modified LDL may induce functional changes in endothelial cells allowing penetration of the endothelium by circulating cells and lipoproteins, or by the direct denudation of the endothelium, accelerating the fatty streak formation.¹³³ Because of these, and other facts described below, oxidised LDL is thought to be potentially more atherogenic than native LDL cholesterol.

As implied above, a large body of epidemiological, biochemical, and experimental animal data exists in support of the hypothesis, and this information has been extensively reviewed.^{260,278} Increased lipid peroxidation products are present in the plasma of patients with occlusive arterial disease. Oxidised LDL is rapidly taken up by the scavenger LDL receptor of macrophages, is chemotactic for circulating monocytes, and is cytotoxic for cells in culture and it can release a chemotactic factor from endothelial cells in culture.²⁶⁰ Experimental animal evidence has indicated that oxidatively modified LDL may occur *in vivo*.²⁷⁸ Also, reasonably secure but not totally conclusive evidence exists in animal models that oxidative modification plays a significant role in atherogenesis.²⁷⁸ Moreover, anti-oxidants inhibit the progression of atherosclerosis in LDL receptor deficient and cholesterol fed rabbits independent of any cholesterol lowering.²⁷⁸ Clearly, sufficient support for this theory exists implicating it as the processing linking the lipoprotein and the cellular hy-

potheses of atherogenesis. Such persuasive support mandates further vigorous study into the oxidative modification hypothesis, especially since *in vivo* information is limited.^{260,278} Indeed, some investigators believe that the available evidence for this hypothesis is sufficient to warrant clinical intervention trials to test the oxidative modification hypothesis.²⁷⁸ As indicated in sections 1.3.3. and 1.6.3, there may also be a connection between this process and some of the thrombogenic aspects of CHD.^{132,134}

1.6.3 The Role of the Platelet in Atherogenesis

Atherosclerosis is a disease process which begins in childhood and is not associated with occlusive arterial lesions until many years later.^{7,9,237} Hence, clinical symptoms appear when the disease is usually at an advanced stage, decades after the onset of the first pathological changes.^{9,242} As discussed, one proposed mechanism of atherogenesis involves the development of vascular endothelial injury resulting from haemodynamic, mechanical, chemical or other factors. This endothelial damage may result in increased localised permeability of the vessel wall and localised adhesion of platelets which release platelet factors that may have a pathological effect in the milieu of abnormal endothelium, exposed intima and abnormal blood flow adjacent to the endothelium.^{242,279} Subsequently, this process could lead to progressive cellular and interstitial changes eventually resulting in atheroma formation. Such a role for platelets in the pathogenesis and evolution of CAD is an attractive and compelling concept that has been the subject of intensive investigation.^{171,280,281}

A role for platelets can also be postulated for the atherogenesis hypothesis involving oxidative modification of LDL. Oxidised LDL interacts with platelets and promotes their aggregation more so than native LDL.¹³⁴ A deficiency of antioxidants may reduce the level of PGI₂ production and lead to the promotion of platelet aggregability.¹³² An important feature of the oxidative modification of LDL hypothesis is that the loss of endothelial cells is not an essential feature as an initiating factor for the promotion of atherosclerosis, although such an outcome could also be caused by modified LDL.¹³³ Therefore, oxidised LDL may initiate endothelial injury and the subsequent sequence of events described in the response-to-injury hypothesis, with the adherence and aggregation of platelets, the release of platelet derived growth factor, the generation of additional growth factors and the growth of smooth muscle cells leading to a thickening of atherosclerotic areas.^{133,242} The platelet, along with lipoproteins and monocytes, would clearly contribute to the pathophysiology of atherosclerosis in such a combination of events.

1.6.4 Other Hypotheses for the Pathogenesis of Atherosclerosis

Quite clearly the formation of atheroma is a complex and incompletely understood process, and numerous other theories have been proposed to explain the formation of atherosclerosis. There is a seminal and comprehensive body of literature supporting a directly active role of lipoproteins in the cellular processes occurring within the vascular wall which may stimulate the formation and progression of atheromatous lesions independent of other processes,^{242,282-286} A monoclonal theory of cellular development of atheroma suggests that atherosclerotic plaque arise from a single line of cell.²⁸⁷⁻²⁹⁰ However, isoenzymatically similar cells may arise late in plaque

formation²⁹¹ and the kind of diet given to induce atherosclerosis may influence the nature of the clonal growth.²⁹² Furthermore, immunohistochemical studies^{293,294} have demonstrated that polyclonal cells occur within the inflammatory infiltrates of atheroma, including both B and T cells.²⁹⁴ The presence of inflammatory cells within atherosclerotic plaques has also emanated in a significant body of literature detailing possible primary immunological²⁹⁵ and infective roles in atherogenesis.^{296,297}

1.6.5 Conclusion

The oxidative modification and the response to injury hypotheses, as primary processes in the development of atherosclerosis, alone or in combination, remain to be proven, as does a role that the platelet may play in atherogenesis. However, clinical evidence suggestive of an interaction between platelets and the arterial wall in the early stage of atherosclerosis does exist.²⁸¹ If some form of platelet dysfunction is involved in atherogenesis, then such a platelet abnormality is likely to be demonstrable at an early stage in the formation of atherosclerotic lesions, that is, during childhood or adolescence. If this were so platelet dysfunction may be detectable in young individuals with a high risk of CAD. Whether increased platelet reactivity is a feature early in the disease process or whether it solely contributes to later pathophysiological processes resulting in the acute thrombotic clinical syndromes is unknown and continues to require critical evaluation. It may even be secondary to underlying vascular pathology and therefore not be involved in the inception or early progression of atheroma. However, this is less likely in view of the current evidence indicating a lack of platelet activation in stable CHD.^{45,74,298}

1.7 THE ROLE OF PLATELETS IN THROMBOSIS

In the following section, the importance of platelets to arterial thrombosis is briefly highlighted since platelets have such a central role in haemostasis,²⁹⁹⁻³⁰¹ and thrombosis plays such an important part in CHD. The thrombotic process results from an interaction between platelets, the vessel wall, the coagulation system and the fibrinolytic system. Normally after superficial endothelial injury only a monolayer of platelets adheres to exposed subendothelium.³⁰² However, thrombus formation can occur following more severe damage following exposure of the components of the vessel wall which activate both platelets and the coagulation system.³⁰² Localised haemostatic plug formation and arterial thrombosis are comparable processes with the same blood constituents responding to vascular injury.²⁹⁹ Platelet aggregation plays a vital role in both physiologic and pathological haemostatic reactions.

1.7.1 Initial Platelet Responses Leading to Aggregation

Primary responses in haemostasis include localised vasoconstriction at the site of vessel injury and platelet adhesion to the exposed sub-endothelial basement membrane and collagen. When platelets adhere to collagen or interact with thrombin there is a profound release reaction of platelet secretory products. Many of the compounds released are platelet aggregating agonists (adenosine diphosphate, thrombin, serotonin) and they can act synergistically to amplify the response of platelet activation to low concentrations of other aggregating agents.²⁹⁹ The effects

of platelets and their secretory and metabolic products on the vessel wall are well documented.^{239,244,303} Platelet dense-granules release ADP, adenosine triphosphate (ATP), calcium, serotonin, magnesium and other substances. The alpha-granules contain important compounds including platelet derived growth factor (PDGF), BTG, PF4, factor VIII related antigen, von Willebrand factor (VWF), albumin, thrombospondin, fibrinogen, fibronectin, antiplasmin, alpha-2-antitrypsin, alpha-2-macroglobulin, as well as chemotactic factors.²⁹⁹ Acid hydrolases are released from the lysosomal granules. Following the release reaction platelet aggregation occurs and with this, phospholipases C and A2 are activated. Phospholipase A2 cleaves arachidonic acid from platelet membrane phospholipid. A combination of phospholipase C, diglyceride lipases and monoglyceride lipases free arachidonic acid from phosphatidyl inositol.²⁹⁹ Activated platelets release potent vasoactive agents such as serotonin, histamine and TXA2.³⁰⁴

Platelet adhesion is an integral step in the maintenance of vascular integrity and is mediated by a number of platelet surface receptors with a high affinity for the adhesive glycoproteins in the sub-endothelium, such as fibronectin and collagen.³⁰¹ It would appear that many of these ligands act on inactivated platelets which, in normal circumstances, are protected from these glycoproteins by the endothelium.³⁰¹ Platelet secretory products, such as ADP and serotonin, and TXA2 manufactured following activation of the cyclooxygenase pathway, are formed locally further enhancing platelet activation.³⁰⁰

1.7.2 Platelet Receptor Function

In addition to the release and production of platelet agonists, platelet receptor exposure is involved in aggregation. Glycoprotein (GP)IIb/IIIa receptors, when activated are exposed and bound by a number of glycoproteins, the most important being fibrinogen.³⁰¹ Fibrinogen and other glycoproteins, such as von Willebrand factor and thrombospondin bind to the GPIIb/IIIa receptor allowing stable platelet aggregates to form.^{305,306} The exposure of the GPIIb/IIIa receptor is the final common pathway fundamental to platelet aggregation.³⁰⁷ The exposure of this receptor is directly augmented by the released contents of platelet storage granules. Further, it is both directly and indirectly enhanced by the formation of TXA2.³⁰⁸ Of particular importance is the fact that all of the platelet agonists can lead to the exposure of the GPIIb/IIIa receptor and subsequently platelet aggregation independent of the cyclooxygenase pathway.³⁰¹

1.7.3 Interaction with the Coagulation System

The adhesion and aggregation of platelets promotes coagulation at multiple stages of the intrinsic coagulation pathway resulting in the deposition of fibrin.³⁰⁹ This thrombotic process can be enhanced when adjacent platelets are activated by thrombin which is formed locally after activation of the coagulation system. Factor Xa and prothrombin are activated following exposure to specific membrane binding sites for coagulation enzymes, zymogens and cofactors.³¹⁰ The binding of activated clotting factors to platelet receptors results in the capacity of platelets to protect factor XIa and Xa from inactivation.³¹⁰ Initiation of the coagulation cascade results in fibrin formation, reinforcing the platelet plug. Normally the fibrinolytic system is then

activated, digesting the haemostatic plug. New growth of endothelial cells of the vessel completes the repair process.^{311,312} Platelets may impair endogenous fibrinolysis as they are a source of factor XIII activity which crosslinks fibrin, reducing its sensitivity to plasmin.³¹³ However, platelets bind plasminogen directly, a process which is augmented after interaction with thrombin and platelet associated fibrinogen,³¹⁴ thus localising plasminogen in the vicinity of platelet aggregates. Nevertheless, platelets also release a plasminogen activator inhibitor³¹⁵ and platelet-rich thrombi appear to be resistant to thrombolysis,³¹⁶ and the process of fibrinolysis influences platelet function, with numerous mechanisms being documented in vivo and in vitro for both inhibition and activation.³⁰¹ Of clinical importance is the activation of platelets associated with pharmacological doses of thrombolytic agents.^{47,49}

Thus, it is clear that activated platelets affect other important components of the haemostatic system. As stated, activated platelets demonstrate enhanced factor Xa activity and prothrombin activation accelerating the formation of thrombin.³¹⁷ Further, aggregation of platelets concentrates activated clotting factors in one localised site. Platelets influence the expression of the fibrinolytic system, having both profibrinolytic and antifibrinolytic effects.³⁰¹

1.7.4 Summary - Platelets and Thrombus Formation

In summary, at least four separate but related pathways result in platelet activation and the formation of stable irreversible platelet aggregates which may subsequently progress to intra-arterial thrombus. Firstly, the adherence to collagen or interaction with thrombin and other platelet agonists induces the platelet release reaction and thence aggregation. This reaction can occur independent of TXA₂ generation.^{300,301,312} However, in normal circumstances, stimulated platelets release arachidonic acid from phospholipids in the platelet cell membrane through the action of phospholipases C and A₂,^{299,311} setting in progress the second major step in the sequence. Cyclooxygenase then converts arachidonic acid to the endoperoxides PGG₂ and PGH₂. Then TXA₂, a powerful stimulator of platelet aggregation, is formed from thromboxane synthetase.^{299,300} Thirdly, the platelet agonists lead to the exposure of GPIIb-IIIa receptors allowing binding of fibrinogen to physically join the platelets to form aggregates. Finally, the formation of fibrin stabilises the platelet aggregates to form a thrombus. Further vessel contraction occurs under the influence of TXA₂ and serotonin. Chemotactic factors released can attract leukocytes to the local thrombus. Subsequently clot retraction consolidates the platelet plug; the thrombus may be incorporated into the wall leading to wall thickening; parts may break away as plasmin lyses the fibrin and platelets deaggregate; or the thrombus may propagate further into the arterial lumen.²⁹⁹ This brief review highlights the importance of the *interaction between platelets and fibrinogen in the formation of platelet aggregates, and this interaction has important implications for the hypothesis of this Thesis.*

1.8 SUMMARY

Atherosclerosis can be viewed as a common source epidemic.³¹⁸ The determinants of common-source epidemics include susceptibility, pathogenesis and virulence of the

agent(s), and the length of the incubation period.³¹⁸ Identification of the susceptible individual has been of prime concern, hence the investigation of risk factors. Despite considerable efforts, still, little is known about the determinants of susceptibility to CAD.³¹⁸

The content of the discussion in this introductory Chapter supports the idea that risk factors may be thought of in terms of being precipitators and/or potentiators of susceptibility to clinical CAD.²⁰² Such a consideration has also arisen from the observations of Cornfield and Detre.³¹⁹ The predisposition to thrombosis and/or platelet aggregation within the coronary circulation could be considered a precipitator and/or potentiator for the acute clinical syndromes. This predisposition could arise from differences in the pathology of the atherosclerotic plaque, functional abnormalities within the arterial endothelium adjacent to plaques, imbalances within the normal mechanisms of intra-arterial thrombin formation and fibrinolysis, local or systemic platelet hyperactivity, or a combination of any number of these possibilities. More recently the existence of factors which are directly or indirectly thrombogenic has appropriately received more attention.⁶⁰

The results of angiographic studies support the concept that CHD progression involves two fundamental processes. The first consists of atherosclerotic lesions progressing to plaque fissuring, platelet aggregation and subsequent healing, with or without minor progression. The second process occurs after plaque fissuring and platelet aggregation, with progression to thrombosis and partial occlusion of the coronary artery, and/or complete occlusion, and/or lysis, and/or thrombus incorporation into the plaque.⁴ In both circumstances the platelet may play a pivotal role.

At present there is unequivocal evidence that the activation of platelets and involvement in thrombosis within coronary arteries occurs in the acute obstructive myocardial ischaemic syndromes. Circulating activated platelets, however, are not a feature of stable chronic CHD, as far as can be currently measured.^{45,298} *Whether more reactive platelets are present in individuals who are susceptible to the development of CAD and/or CHD remains to be elucidated.* Mural thrombosis over atherosclerotic plaques would appear to be an ongoing process without necessarily any clinical consequences at the time.²³⁸ Why an individual progresses to an occlusive arterial event is unknown but probably results from numerous interacting components, one possibility being the presence of genetically derived and/or environmentally induced hyperactive platelets given the appropriate stimuli.

Heightened platelet reactivity may be important in platelet involvement in the early stages of atherosclerosis or the later clinical manifestations. If such platelet dysfunction does exist, it may require intermediary pathophysiological changes to become manifest. Such a proposal accommodates the concept of an interaction between the environmental "triggers" and the presently unaccounted for inheritance factor. Another possibility is that part, or all, of the familial predisposition to CAD is related to an abnormality in platelet function. Therefore an evaluation of platelet function in young individuals at increased risk of the development of CAD and in those manifesting their susceptibility by the clinical manifestation of the disease is of value. The thesis that thrombosis is an active component in the development of CHD and CAD, and is unrelated to chance requires further clarification. This is particular-

ly important since an understanding of postulated thrombogenic factors, whether environmental and/or heritable, may have important implications for identifying susceptible individuals more accurately, thereby improving methods of prevention and enabling implementation of more definitive treatment.

1.9 THE QUESTION ASKED IN THIS THESIS

1.9.1 The Basic Question

Do individuals with premature CHD have a platelet abnormality resulting in hyper-responsiveness to physiological and/or pathological stimuli, and, if so, is such an abnormality a primary factor or produced by other influences?

1.9.2 Why Ask the Question ?

The foregoing discussion provides the rationale as to why this question is important and requires evaluation. The salient issues can be summarised as follows:

1. Some evidence exists implicating increased platelet reactivity in patients with CHD and in those with some of the important risk factors. Evidence indicating that platelets are quiescent between acute events does not exclude the possibility of platelet hyper-responsiveness upon stimulation or provocation *in vivo*.

2. An unequivocal role of platelets in the acute thrombotic occlusive clinical syndromes of CHD has been established. However, the relative importance of platelets as an initiator and/or potentiator of thrombosis in these events is unknown.

3. Coronary angiographic data indicates that atherosclerotic plaques enlarge rapidly and sporadically. This may be due in part to mural thrombosis causing sudden rapid progression of lesions. Another reason for such changes may also be due rapid accumulation of modified LDL and foam cell formation within an atherosclerotic plaque.

4. Pathological studies provide persuasive evidence for platelet-fibrin deposition in intramural lesions.

5. The presence or absence of CHD does not simply relate to the extent of coronary atherosclerosis.

6. Current risk factors do not account for all cases of CAD or CHD. They also cause premature disease in only a minority of individuals with one or more risk factors.

7. The importance of abnormal levels of certain circulating lipoproteins cannot be under valued and seem to be a necessary prerequisite for the development of the early stages of atherosclerosis. However, abnormal blood lipoproteins do not have a critical influence on the expression of the clinical manifestation of atherosclerosis, and the presence of lesions does not inevitably lead to CHD.

8. Premature CHD has a familial component which remains incompletely explained by the heritability of conventional risk factors.

9. The influence of thrombogenic risk factors on the expression of CHD is only now being appropriately evaluated and defined. Indeed, a large body of biological evidence already exists indicating an important role of platelets in atherogenesis.

The relevance of addressing the question stated is substantiated by the foregoing discussions and the outcome will contribute to the currently developing model for the pathogenesis of CHD.

1.10 THESIS FORMAT

The main study was a case-control study of males, aged less than 50 years, with premature CHD, and an age, sex and socioeconomically matched control group without CHD. Because of major potential confounding factors and areas of correctable bias, a number of additional studies were undertaken.

Firstly, a group of male subjects with known CHD were evaluated to determine if case group subjects needed to be separated into those with and without active myocardial ischaemia, as assessed by exercise testing. This group was also used to determine if it was essential to select subjects in the CHD case group who could have their cardiac medications stopped. That is, whether continuation of cardiac medications altered the *in vivo* measures of platelet function that were used in the case-control study.

Secondly, the major lipoproteins were evaluated in greater detail because of the important potential for confounding the results by their influence on platelet function. Furthermore, the evaluation of the current diet and alcohol intake for both the case and control group was performed given the potential influence of dietary factors on platelet function.

Thirdly, the fibrinogen level may have a major effect on platelet function. This necessitates a detailed evaluation of this risk factor, particularly in relation to platelet function tests.

In addition, a number of "new" CHD risk factors have come into prominence over recent years. These risk factors may have a potential confounding influence on the case-control study proposed and the use of platelet function tests. The "new" risk factors were evaluated in more detail, using another study population prior to the case-control study, were specific measures of hostility and other behavioural measures, central body obesity and blood glucose levels, and plasma fatty acid content. The influence of these factors was evaluated because of their possible confounding effect on the results. These factors and their interaction with platelet function measures are discussed in Appendices 1,2 and 3.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

General methods and materials are described in this chapter. Specific methods are described in appropriate chapters.

2.2 MATERIALS

2.2.1 General

All glassware was washed in distilled deionised water and further cleaned by soaking in Pyroneg solution, then scoured with a brittle brush, followed by extensive rinsing with tap water and then at least two rinses with distilled water. Glassware was then oven dried at approximately 80°C. Apart from platelet aggregation cuvettes and for the incubation of whole blood for serum TXB2 measurement, glassware was not used with blood, platelet rich plasma (PRP) or platelet poor plasma (PPP). The aggregation cuvettes were treated in a solution of chromic acid overnight and then washed as described above. The clean cuvettes were soaked for pretreatment with siliconising solution (Coatasil) for 2 min, then drained and washed with distilled water, and drained overnight. Syringes used were all polypropylene (Terumo).

2.2.2 Thromboxane B2 Assay Materials

2.2.2.2 Tris gelatin buffer. Stock Tris buffer 150 mM, pH 7.4 stored at 4°C was diluted 1:10 and 500mg/litre of gelatin was dissolved by stirring while warmed to approximately 37°C.

2.2.2.3 TXB2 antibody. The antibody (from Seragen for the assays in the studies described in Chapter 4 and Advanced Magnetics Inc, Cambridge MA, USA, for the remaining assays) was diluted in Tris gelatin buffer and stored in aliquots at -20°C for use in later assays.

2.2.2.4 TXB2 Standards. The TXB2 standards (Upjohn, Kalamazoo, Mich, USA) were dissolved in Tris gelatin buffer and divided into aliquots of 100ng/ml and stored at -20°C prior to use in batch assays.

2.2.2.5 ³H-TXB2 antigen. The radiolabelled antigen (tritiated TXB2, 200 curies/mmol) was supplied by Amersham, UK. The specific radioactivity of the antigen was initially 25 microcuries in 250 μ l. This was diluted to give a stock solution of 0.02 microcuries/ μ l. Between 10 to 15 μ l of this stock solution was diluted with 2.5 ml of Tris gelatin buffer to give approximately 5000 cpm for the total count of the working solution.

2.2.2.6 Dextran coated charcoal. To 10ml of Tris gelatin buffer were added 10mg dextran, 10mg bovine serum albumin and 60mg charcoal, or the equivalent proportion. They were mixed by stirring on ice for 10 min.

2.2.2.7 Scintillant. Five ml of xylene and triton in aqueous solution was added to the supernatant prior to counting in the beta-counter.

2.2.3 Materials for Platelet Aggregation Studies

2.2.3.1 **Citrate anticoagulant.** The citrate solution was 0.1 M trisodium citrate at pH 7.4 and stored at 4.0°C as a stock solution.

2.2.3.2 **Reagent Diluent.** Reagent diluent (RD) was diluted from stock solutions. Stock I RD consisted of 160 gm sodium chloride, 4 gm potassium chloride, 20 gm sodium hydrogen carbonate and 1g sodium dihydrophosphate dissolved in one litre of distilled water. Five ml of stock I was added to 100 ml of distilled water and the pH adjusted to 7.4 to be used as the working RD for the day.

2.2.3.3 **Alcohol.** Pure alcohol (May and Baker Pty Ltd, Victoria, Australia) was used to stop the platelet aggregation response by addition (10ul) to the aggregation cuvette at a predetermined time.

2.2.3.4 **Adenosine diphosphate.** Adenosine diphosphate (ADP), grade 1 sodium salt (Sigma Chemical Co, St Louis, Mo, USA), was stored at -20°C at an initial concentration of 10mM and diluted in RD at -4°C to the required dilution prior to use as a platelet aggregating agonist.

2.2.3.5 **Collagen.** Lyophilised soluble calf skin collagen (Bio/Data Corporation) at a concentration of 1.9mg/ml was stored at 4°C prior to reconstitution with distilled water, pH 7.0. Following reconstitution it was stored at 4°C for a maximum of 30 days.

2.2.3.6 **Adrenaline.** Adrenaline bitartrate salt (Sigma Chemical Co, St Louis, Mo, USA) was stored in aliquots of 10mM at -20°C and diluted in RD at -4°C to the required concentration prior to use as a platelet aggregating agonist.

2.2.3.7 **Platelet rich plasma (citrated).** Nine ml of blood was collected into 1ml of sodium citrate (0.1M trisodium citrate, pH 7.4, 4.0°C). The PRP was prepared by centrifugation at 100g for 15 min. The upper 2/3 of the supernatant was separated and stored air free at 37°C in stoppered syringes. PRP was used within 2 hr of sampling.

2.2.3.8 **Platelet poor plasma (citrated).** After separation of PRP, the remaining fraction was centrifuged at 8000g (Eppendorf Microcentrifuge 3200, Eppendorf Instruments Ltd, Hamburg, West Germany) for 2.5 min and the upper 2/3 of this supernatant was collected as PPP.

2.2.4 Platelet Count Ratio Method Materials

2.2.4.1 **EDTA-Formaldehyde solution.** This solution contained sodium chloride (0.14 M) potassium hydrogen phosphate (0.01 M), sodium-EDTA (0.001 M) and formaldehyde (1% vol/vol) in distilled water and adjusted to pH 7.36.

2.2.4.2 **EDTA solution.** Sodium chloride (0.35 M) and sodium EDTA (0.001 M) were dissolved in distilled water and adjusted to pH 2.26.

2.3 THROMBOXANE B2 ASSAY METHODS

2.3.1 TXB2 Collection and Storage

Blood was collected after a 12 hr fast, with the patient rested and supine, and with minimal venous stasis. Using a 19 gauge "butterfly" needle with a 15 cm plastic tube, 9ml of blood was collected into a syringe containing 1ml of ice-cold anticoagulant (EDTA 10mM, PGE1 1ug/ml) and kept on ice until PPP was prepared within 15 min (2000 rpm for 60min). The middle 1/3 of the PPP fraction was frozen at -70°C prior to batch processing

Blood for serum TXB2 measurement was collected into a plastic syringe and 1ml aliquots were immediately transferred into glass tubes for incubation at 37°C for 60 min. Serum was separated (2000rpm for 5 min) and stored at -70°C prior to batch processing.

2.3.2 TXB2 Assay Procedure

The radioimmunoassay (RIA) is based on a competitive reaction between TXB2 and ^3H -TXB2 for a limited number of binding sites on a TXB2 specific antibody. The amount of ^3H -labelled TXB2 bound by the antibody will be inversely proportional to the concentration of unlabeled TXB2 present in the plasma or serum samples. The antibody bound ^3H -TXB2 is separated by precipitation with dextran coated charcoal solution. After centrifugation the radioactivity of unbound ^3H -TXB2 remaining in the supernatant is measured in a beta counter (Packard Tri-Carb 460 liquid scintillation counter, Packard Instrument Co, Downers Grove, Ill, USA). A standard curve is constructed by measuring the proportion of ^3H -TXB2 unbound and remaining in the supernatant in the presence of the TXB2 standards which have been precisely diluted from a known standard. The standard values are plotted on logit graph paper with a range between 4pg per sample to 250pg per sample. The unknown samples were diluted to fit within the linear section of the standard graph. All samples and standards were assayed as duplicates and the results averaged. If a large discrepancy occurred, that sample was re-assayed. The volumes and order of addition followed a specific format, which was always observed and was as follows:

	Buffer* or sample (ul)	Standard (ul)	^3H -TXB2 (ul)	TXB2 Ab (ul)
TC	900	-	100	-
NSB	200*	-	100	-
Bo	100*	-	100	100
Standards	-	100	100	100
Samples	100	-	100	100

TC = total count, NSB = non-specific binding, Bo = zero binding, tracer = ^3H -TXB2, Ab = antibody

Following addition to the assay tubes (3DT tubes), the combination was incubated for 2 hr at 25°C before being immediately centrifuged for 15 min at 4°C and 1000g. Without disturbing the sediment, the supernatant was decanted from each tube into

scintillation vials immediately after centrifugation. A fixed volume (5ml) of scintillant fluid was added to each vial and counting was performed in a liquid scintillation counter. The approximate total count was 5000 cpm, the non-specific binding (NSB) was usually less than 1/20 of the total count, and the zero binding ranged between 40-50% of the total count. The seven standards used for the standard curve were 250, 125, 63, 32, 16, 8 and 4 pg/100ul of solution.

2.4 BETA-THROMBOGLOBULIN ASSAY

2.4.1 Introduction

Beta-thromboglobulin (BTG) is released from platelet alpha-granules following activation and degranulation of the platelet. The presence of BTG in plasma has been regarded as an *in vivo* marker of platelet activation.^{76,77,88,320-324} BTG has been reported to be present in increased concentrations in plasma of patients with various vascular disorders.^{76,77,321,323}

2.4.2 Beta-thromboglobulin Measurement

BTG was measured with a RIA kit (Amersham International) developed for the immunoassay of human BTG in PPP. The range covered by the assay is 10-225ng/ml. BTG levels in normals using this kit is approximately 26ng/ml with 95% of values being below 52ng/ml. Each batch of BTG RIA kits has a within assay coefficient of variation (CV) less than 7.5% on a control plasma value of 45ng/ml. The between assay CV of kits from 2-14 weeks was found to be 9.9%, 10.7% and 15.3% in three different laboratories.

The RIA method depends on competition between BTG and ¹²⁵I-labeled BTG for a limited number of binding sites on a BTG specific antibody. The amount of ¹²⁵I-labeled BTG bound by the antibody will be inversely proportional to the concentration of unlabeled BTG present in the PPP samples. The antibody bound ¹²⁵I-labeled BTG is separated by precipitation with an ammonium sulphate solution. After centrifugation and removal of the supernatant, the precipitated radioactivity is measured in a gamma counter. A standard curve is constructed by measuring the proportion of ¹²⁵I-labeled BTG bound in the presence of a series of BTG standards which have been calibrated against a primary BTG standard (Amersham) and range from 10-225ng/ml. All samples and standards were assayed as duplicates and the results averaged. If a large discrepancy occurred, that sample was re-assayed in a subsequent batch run.

2.5 PLATELET COUNT RATIO METHOD

2.5.1 Background

The platelet count ratio (PCR) refers to the ratio of the difference in platelet counts between platelet-rich plasma separated from blood anticoagulated with EDTA and EDTA-formalin. Platelet aggregates can be stabilised by withdrawing whole blood

into formaldehyde solution which protects them from the deaggregatory action of EDTA.^{325,326} Deaggregation is a function of the calcium chelating ability of the EDTA, and the EDTA also prevents *ex vivo* aggregation and clotting. Nevertheless, the PCR may reflect platelet aggregate formation during collection and hence may give a measure of *ex vivo* platelet reactivity.

2.5.2 Platelet Count Ratio Determination

The PCR was determined by a modification of the method of Wu and Hoak.³²⁵⁻³²⁸ Using minimal initial venous stasis, and following a clean venepuncture with a 19G "butterfly" needle free flowing venous blood was obtained without a tourniquet. The initial 0.5ml of venous blood was discarded and one ml blood samples were collected into separate syringes containing 4 ml of either buffered sodium EDTA solution (sodium EDTA solution 0.001 mol/litre, pH 7.26) or buffered sodium EDTA-formaldehyde solution (formaldehyde 1% wt/vol, sodium EDTA 0.001 mol/litre, pH 7.36). The solutions and blood were mixed by gentle inversion. Aggregates disperse in the former and are fixed in the latter. Following 15 min incubation at room temperature, the samples were centrifuged in plastic tubes at 100g (800 rpm) for 10 min, at 22°C. Platelet counts were determined in the supernatant with a Coulter Counter (model ZF). The PCR was calculated as the ratio of the platelet count in EDTA-formaldehyde solution over that in the EDTA solution. The first syringe contained 4ml of EDTA solution and the second 4ml of EDTA-formaldehyde and triplicate samples were taken in the same order and the three averaged. If one sample was discordant then the process was repeated.

2.6 PLATELET AGGREGATION METHODS

2.6.1 Background

Platelet aggregation was studied employing commonly used spectrophotometric methods.^{329,330} Platelet aggregation was studied with a dual channel aggregometer (Payton Dual Channel Aggregation Module, Payton Associates, Ontario, interfaced with a Riken-Denshi SP-H6P Chart Recorder, Riken-Denshi Co Ltd, Japan).^{329,330} The number of platelets in the PRP was determined with a Coulter Counter. The baselines for light transmission 10% to 90% were set with PRP and PPP respectively. Silicone-coated stir bars (1 by 5 mm) were added to siliconised glass cuvettes warmed to 37°C with the stir speed at approximately 900 rpm. Aggregation was measured as an increase in percent light transmission.

The epidemiological aspects of *in vitro* aggregability of platelets has been evaluated in the Northwick Park Heart Study.³³¹ Aggregability increases with age and fibrinogen levels, is less in women than men at all ages (being partly accounted for by differences in haemoglobin and haematocrit), varies in races with white races having more aggregability than black, is less in male smokers compared with non-smokers, and there is a mild relation between increased alcohol intake and reduced aggregability.³³¹

Aggregation is an *in vitro* measure of platelet function, following exposure to

specific agonists. In this study, the agonists used were collagen, adrenaline and ADP (see sections 2.2.3.4, 2.2.3.5 and 2.2.3.6).

2.6.2 Platelet Aggregation Quantitation

Following blood collection into a 10ml syringe containing 1ml of sodium citrate, the syringe was sealed and placed on ice until PPP and PRP were prepared within 15 min of sampling (see 2.2.3.7 and 2.2.3.8).

2.6.2.1 LT50. The lag time to 50% of maximal alteration in light transmission (LT50) measured in seconds.³³²

2.6.2.2 Maximum rate of change in light transmission (R). The maximal rate of change in light transmission was determined from the maximum slope of the aggregation curve.³³³ The maximum rate of aggregation for collagen, ADP and adrenaline will be abbreviated as RCOL, RADP and RADR respectively.

2.6.2.3 Threshold concentration. The lowest concentration of aggregating agent able to produce greater than 50% second phase aggregation.

2.6.3 Standardisation of Aggregation Methods

Previously it has been considered that platelet aggregation tests using ADP and adrenaline were most likely to differentiate between hypersensitive and "normal" control platelets.³³⁴ However, there are numerous factors which must be standardised for *in vitro* testing, including experimental conditions, intrinsic and extrinsic factors associated with platelet hypersensitivity, dietary fats, other disease states and other factors.^{77,311,312,334-337} To endeavour to standardise the conditions under which aggregation methods were used, the criteria described below were adhered to for all studies.³³⁶

(a) *Controls and subjects.* Age and sex matched controls were used as there is a possible age-dependent enhancement of platelet responses to collagen.³³⁶ Sex matched controls are required because the haematocrit affects the concentration of citrate and hence Ca^{++} .³³⁶ The subjects needed to be fasting and non-smoking for 12 hr.

(b) *Plasma handling.* Plasma was kept in a polystyrene syringe to prevent activation of factor XII, the syringe was stoppered to prevent loss of carbon dioxide and subsequent increase in pH of the plasma, and the plasma was stored at 37°C in a water bath.

(c) *Timing in the protocol.* The time from venepuncture until aggregation tests for the patients and controls was standardised between 1 and 2.5 hr, the centrifugation was standardised (100g for 15 min) and was carried out within 15 min of blood collection.

(d) *Platelet count.* Plasma samples also had a platelet count routinely performed. Differences in platelet aggregation are negligible if the platelet count is maintained over a range 200,000 to 300,000/ m^3 .³³⁶

(e) *Aggregometer.* The aggregometer was calibrated before each test by setting the reading to 10% for PRP and 90% for the PPP. The sample holder was set to a

temperature of 37°C, the PRP was stirred at a constant rate of 900rpm, and the length, width and consistency of the stir bars were the same.

(f) *Storage of platelet rich plasma and the effect of time.* The results of platelet function tests, including platelet aggregation, are dependent on the time the samples to be tested are stored. When platelets are stored as PRP there is an initial increase in aggregation^{312,346,350,351}. The changes in platelet aggregation with time are thought to be due to intrinsic changes within the platelet rather than externally mediated effects from plasma³⁴⁶. Nevertheless, the potential for platelet aggregation to decrease with time after collection of blood and when stored at 37°C³⁴⁷. Therefore, the time which the PRP was left standing prior to aggregation was standardised to between 1 and 2.5 hours. The order in which the tests were performed were also standardised for each agonist and was the same in both groups.

2.6.4 Collagen-induced aggregation

2.6.4.1 **Background.** Collagen induces platelet adherence, the release reaction, platelet aggregation and thromboxane production. Collagen-induced aggregation is thought to be mainly due to the action of ADP and TXA2 on platelets.³³⁶ However, other additional factors would also appear to be involved, including additional pathways for dense granule secretion in the initial response,³³⁸ the presence of albumin on the platelet surface,³³⁹ the presence of leukocytes and fibrinogen,^{340,341} the interaction of red cells,³⁴² and possibly other alternate pathways.^{338,341}

The collagen for *in vitro* platelet aggregation needs to be a suspension of collagen fibrils or acid soluble collagen.³³⁶ Shape change occurs within 15-20 sec of contact, and a subsequent lag phase dependent on the concentration and mixing of the collagen with platelets, as well as the responsiveness of the platelets.³³⁶

2.6.4.2 **Method.** To detect conditions in which collagen-induced aggregation is enhanced, a concentration of collagen that causes less than maximum aggregation of control platelets must be used.³³⁶ In this study an amount of collagen that caused less than maximal aggregation was documented. This was determined by titration of the collagen from a concentration known to invariably cause aggregation and the PRP samples were then exposed to decreasing amounts of collagen until no aggregation was observed.

The concentration of collagen used was 1.9 ug/ul, and the starting volume was 25 ul added to 500 ul PRP giving a final concentration of 90 ug/ml PRP. This final concentration of collagen always caused maximal aggregation. The added volumes of collagen to the PRP were:

Volume	Final Concentration in PRP
25 ul	90 ug/ml
20 ul	73 ug/ml
15 ul	55 ug/ml
10 ul	37 ug/ml
5 ul	19 ug/ml

2.6.5 ADP-Induced Aggregation

2.6.5.1 Background. After exposure to ADP platelets undergo shape change, and in the presence of appropriate conditions (temperature, pH, carbon dioxide and calcium concentration³⁴³⁻³⁴⁵), concentration of ADP and fibrinogen, platelets can be induced to aggregate. The results of platelet function tests depend on the time interval between blood collection and the test. There is an increase in aggregation with time³⁴⁴ which is a platelet related effect and not plasma related.³⁴⁶ Others, however, have found ADP-induced platelet aggregation may decrease.³⁴⁷

Recent evidence in animal models suggests that ADP is involved in activating platelets *in vivo*.³⁴⁸ At sites of vascular injury, enough ADP is released from damaged cells to induce platelet aggregation.³⁴⁸ In humans there is substantial evidence that ADP is released from red cells and induces activation and aggregation of platelets³⁴⁹ and is influenced by flow conditions.³⁴⁹ Furthermore, ADP generated in whole blood *ex vivo* triggers spontaneous aggregation of platelets.³⁴⁹ Indeed, there is a large body of *in vitro* and *ex vivo* evidence for the dependence of the haemostatic aggregation of platelets on free ADP in blood.³⁴⁸

2.6.5.2 Method for ADP-induced aggregation. A series of decreasing concentrations of ADP (25 μ l) were added to 500 μ l of PRP. Maximal platelet aggregation was achieved by adding 25 μ l of ADP at a concentration of 1.0 mM (added concentration). Decreasing concentrations of ADP in increments were then added to determine the threshold concentration for ADP-induced aggregation. The order of addition of these concentrations was (added concentration ADP) 1000.0 μ M, 100.0 μ M, 50.0 μ M, 25.0 μ M and 12.5 μ M.

2.6.6 Adrenaline Induced Platelet Aggregation

2.6.6.1 Background. Platelets have approximately 100 alpha-receptors on each cell surface and binding of adrenaline promotes platelet aggregation.³¹² In citrated-PRP, adrenaline causes aggregation and the release of platelet granule contents, although such reactions do not occur in PRP that has been anticoagulated with hirudin.³⁵⁰ The response of human platelets to adrenaline is influenced by conditions of aggregation such as calcium concentration, the presence of fibrinogen and the time PRP is allowed to stand at room temperature prior to stimulation with an agonist.^{312,350,351}

Subthreshold aggregatory concentration of ADP or collagen, which are in themselves insufficient to cause platelet aggregation, may do so with the addition of adrenaline.³¹² Indeed, adrenaline potentiates aggregation induced by a number of agonists.^{70-72,337,352,353} However, fibrinogen is not required for adrenaline-induced potentiation of ADP-induced aggregation.³⁵⁰ There is also considerable variability in the responsiveness to adrenaline of platelets obtained from normal subjects.^{354,355}

2.6.6.2 Method for Adrenaline-induced aggregation. A series of decreasing concentrations of adrenaline (25 μ l) were added to 500 μ l of PRP. Maximal platelet aggregation was attained by adding 25 μ l of adrenaline (added concentration of 1.0 mM). Decreasing concentrations of adrenaline were then added to determine the threshold concentration for adrenaline-induced aggregation. The order of addition of these concentrations was the same as for ADP.

2.7 PLATELET COUNT

2.7.1 Method

Platelet numbers in peripheral blood collected in an EDTA coated tube were measured with an automated Coulter Counter. The numbers are expressed as 10^9 /litre.

2.7.2 Quality Control

Blood cell quality control methods employed stabilised cell suspensions of human and fixed human and avian erythrocytes and stabilised mammalian platelets in a plasma like fluid with preservative (Diff-trol 8 plus, Hematology Control, Abnormal High and Abnormal Low, American Dade, American Hospital Supply Corporation). This product was used in the quality control for the determination of red and white cell counts (WCC), haematocrit (Hc), mean red cell volume (MCV), haemoglobin (Hb), red cell distribution width (RDW), platelet count and MPV.

2.8 PLATELET VOLUME

2.8.1 Method of Measuring Platelet Volume

When particle shape, electrolyte resistance, electronic amplification and capillary aperture diameter are kept constant, a Coulter Counter produces electrical impulses whose amplitude are proportional to the volume of the individual particles passing through its aperture.³⁵⁶ From the discussion in section 6.1, it is clear that standardisation of procedures for collection and processing of blood to measure MPV is essential given the plethora of potential confounding factors related to the methodology. Therefore, in this study the collection time was always between 9 and 10 am, a large bore needle (19G) was used, blood was collected with minimal haemostasis, the same equipment and procedure was always used and the same individual always collected the blood. The anticoagulant was EDTA and the blood was processed within 3 to 5 hours after the collection. Prior to processing, the blood was stored at a constant ambient room temperature. The same Coulter counter S-plus was used for all the platelet count and MPV measurements. Blood for MPV measurement was collected in tubes containing EDTA and was processed within 2 hr of collection.

Blood cell quality control was performed using stabilised cell suspensions (Dade, Diff-Trol 8 Plus for high levels and Dade, Diff-Trol 8 Plus for low levels). This reagent consists of stabilised human and fixed human and avian red cells and stabilised mammalian platelets in a plasma-like fluid containing preservative. The MPV (in fl) for the reagent using a non-azide diluent is 9.0 with a range of 7.5 to 10.6 fl. The measurement was undertaken in the Clinical Haematology service laboratory at Woden Valley Hospital, Woden, ACT.

2.9 METHODOLOGY FOR OTHER LABORATORY MEASURES

2.9.1 Dyslipoproteinaemia

Blood (fasting state) was obtained for measurement of total cholesterol, triglycerides and high density lipoprotein cholesterol (HDL). Measurement of these parameters was undertaken in a research laboratory (Prof Leon Simons, University of NSW, St Vincents Hospital, Sydney) with established high quality control of sample measurement and precision of measurement.³⁵⁷⁻³⁵⁹ Concomitant cholesterol measurements were made in a service laboratory (Woden Valley Hospital Department of Biochemistry, Woden, ACT), the blood being taken simultaneously. The plasma cholesterol was measured by a standard enzymatic colorimetric test in kit form (Roche Diagnostics, MA kit 30). Triglycerides were also measured by a standard enzymatic determination of glycerol after enzymatic hydrolysis of triglycerides (Roche Diagnostics, s, MA-kit 10). The HDL was measured in serum after non-HDL cholesterol fractions were separated by precipitation with heparin and manganese chloride.

Serum cholesterol was measured by carrier-bound technology on a Kodak Ektachem 700 analyser, based on the enzymatic method previously described.³⁵⁷ After hydrolysing cholesterol esters to cholesterol, hydrogen peroxide is generated from the catalysed oxidation of total cholesterol. The peroxide oxidises a leuco dye in the presence of peroxidase to produce a dye having maximal absorption at 670 nm. The density of the dye, being proportional to the concentration of cholesterol, is measured by reflectance spectrophotometry at 540nm.

Apolipoproteins A1 and B were analysed by peak rate nephelometry on a Beckman ARRAY Protein Analyser.³⁶⁰ In the reaction, antibody to human apolipoprotein is exposed to the apolipoprotein and the increase in light scatter resulting from the antigen-antibody interaction is converted to peak rate signal which is a function of the apolipoprotein concentration in the sample. Following calibration, the peak rate signal for the particular assay is converted to concentration units by the analyser. Coefficients of variation values of <5% for within run precision and <8% for between run precision for both apolipoproteins was obtained by replicate testing of 59 control samples.

The service laboratory participates in an internal external quality control programme and has regularly ranked in the top twenty laboratories in Australia for both accuracy and precision. External quality control methods employed demonstrated within laboratory coefficient of variation for the measures to be less than 5%. Internal quality control for the laboratory is run daily and coefficients of variation for cholesterol of 2% or less is obtained at both low (2.5 mmol/l) and high values (6.5 mmol/l).

Blood was collected from the same venepuncture for the measurement of serum cholesterol in separate laboratories, and plasma cholesterol in one of the laboratories. The blood was collected for all patients in the morning (between 0830 and 1030 hrs), after an overnight fast (>12 hrs), in the supine position and with minimal (<2 min) venous occlusion. The specimen was kept on ice and processed within 2 hr. The plasma samples, which were collected in heparinised tubes, were analysed on

the same day in the local laboratory; the serum samples for the second laboratory were stored at -20°C for 1 to 2 weeks prior to shipping for analysis; and the local laboratory serum specimens were stored at -70°C for <12 months prior to analysis. The specimens for analysis in the research laboratory were shipped overnight in ice within a temperature-controlled container. These methods are as recommended to minimise variance incurred through sample collection, handling and shipping.³⁶¹

Assessment of these measurements and the quality control is provided in Chapter 5.

2.9.2 Blood Glucose Level

The fasting blood glucose level was measured for each patient as a screening test to determine overt glucose intolerance. All cases of glucose intolerance will not be detectable by this method. This measurement was performed in a service laboratory (Woden Valley Hospital, Department of Biochemistry, Woden, ACT).

2.9.3 Fibrinogen

Fibrinogen was measured by the heat precipitation method.^{362,363} Fibrinogen was selectively precipitated at 56°C, and the optical density (measured as absorbance) of the plasma was subsequently quantitated at a wavelength of 540nm to allow calculation of the fibrinogen level. The test was standardised routinely once per month, and whenever fresh buffer was prepared. Standardisation was performed using a reference plasma assayed for fibrinogen which was at a known concentration. This was performed in a service laboratory (Coagulation Laboratory, Department of Haematology, Woden Valley Hospital, Woden, ACT).

2.9.4 Glycosylated Haemoglobin Estimation

The colourimetric method for the estimation of haemoglobin glycosylation was based on the principles developed by Fluckiger and Winterhalter³⁶⁴ and modified by Parker and colleagues.^{365,366} Subramanian and coworkers utilised further modifications by separating the globin chains prior to the assay with acid-acetone extraction.³⁶⁷ However, the more simple method used demonstrates both very good reproducibility (%CV=3) and comparability with high performance liquid chromatographic methods.³⁶⁶ The method does not depend upon altered physical properties of the haemoglobin (Hb) molecule, but rather on estimated ketoamine-linked hexoses directly.^{364,368} Ketoamine linked hexoses on the glycosylated haemoglobin (HbA1C) are converted to hydroxymethylfurfural (HMF) by limited hydrolysis in a weak acid,^{364,368} and the HMF is then transformed to a coloured complex with thiobarbituric acid.³⁶⁴ Thus, HbA1C is boiled in phosphoric acid (2.5M) resulting in the liberation of 5-HMF. The coloured compound derived from the reaction with thiobarbituric acid was measurable photometrically at 443 nm. The units were expressed in nmoles of HMF/10mg of Hb and the reference range was 5.0 to 10.0 nmol HMF/10mg Hb. This method measures total glycosylated proteins and therefore the erythrocytes were carefully washed to remove plasma prior to the preparation of the hemolysate.³⁶⁶ Intracellular proteins will be present in the hemolysate and are measured along with the glycosylated hemoglobin.³⁶⁶ This measurement was performed in the Department of Biochemistry, Woden Valley Hospital, Woden, ACT.

2.10 METHODS FOR DOCUMENTING CLINICAL RISK FACTORS

2.10.1 Hypertension

Indirect blood pressure (BP) measurement was made with a cuff and mercury sphygmomanometer. The pressure was measured in the prone position whilst fasting, without restricting clothing, and in a similar ambient temperature. Systolic pressure is the first perception of sound, and the diastolic pressure is the loss of perception of sound (Korotoff phase V). Although intra-arterial measurements will be obtained at the time of the catheterisation, these were not used because of inherent variables affecting the BP at the time of, and related to, cardiac catheterisation.

The prior diagnosis of hypertension, the time since diagnosis of hypertension, and the period of time treatment has been administered was documented. An electrocardiograph (ECG) was obtained and the presence of left ventricular hypertrophy was determined by standard criteria.³⁶⁹

2.10.2 Tobacco Intake

The current use or non-use, time of onset, time of cessation if applicable, usual number of "tailor" made used daily, and the equivalent "pack years" were documented. The pack years is the equivalent number of years of smoking 20 cigarettes per day. For example, an individual smoking 40 cigarettes per day for 10 years has a 20 packyear smoking history.

2.10.3 Family Medical History in First Degree Relatives

The first degree family history was documented on historical grounds. The age of diagnosis, time of death if applicable, whether this was due to CHD, and the presence of other risk factors in the family members were recorded.

2.11 METHOD FOR ANGIOGRAPHIC ASSESSMENT

The severity of CAD can be measured semi-quantitatively by the grading of coronary angiograms. A distinction between diffuse atheromatosis and severity of discrete lumen stenosis can be made by separate scoring methods.²⁰⁷ These scoring methods have previously been independently validated.²⁰⁷ The same internal consistency of the scoring system against an alternative scoring method has been validated in this study. More importantly, the intra-observer error (reproducibility and reliability) for the method used was acceptable, the coefficients of variation ranging between 4% and 9% for the main angiographic scores used in the Thesis.²⁹⁸ The methods used and the validation of the methods are described in detail in Chapter 8.

2.12 EXERCISE TEST PROTOCOL

All subjects who had exercise tests were fasting. The exercise convention used was the Bruce protocol which is a maximum graded treadmill exercise test in the upright

position. All exercise tests were performed at approximately the same time of day, between 9.00 and 11.00 a.m., and following an initial 1/2 hr supine rest period. The electrocardiogram was continuously monitored and rhythm strips recorded every minute, at the onset of angina, and until any ECG changes returned to the resting state. The criteria for an ischaemic ECG response, when compared with the baseline tracing, were either: (1) horizontal or downsloping ST segment depression of 1 mm or more; or (2) > 2mm upsloping ST segment depression at 80 msec after the J point for 3 consecutive complexes.

2.13 CASE-CONTROL METHODOLOGY

2.13.1 Definition of a Case-Control Study

A case-control study is an example of an observational analytic epidemiological investigation which can provide a qualitative description of a particular disease in a specific population at a given time, and allowing quantitative assessment of characteristics within the population in relation to the disease. Subjects are selected on the basis of whether they do (cases) or do not (controls) have the disease under study.³⁷⁰ The discussion on the choice of methodology and design is given in Chapter 4.

2.14 GENERAL STATISTICAL ANALYSIS

The following section describes the methods employed for the case-control study of young males with CHD, which is the main component of this study. Additional statistical methods, when employed, will be described in the appropriate section.

2.14.1 Data Management

The results were stored on the Australian National University's previous VAX "main frame" computer for statistical analysis using the Statistical Package for Social Sciences programme. Subsequently, these programmes were transferred onto a Sun Computer and accessed through a Sun Workstation.

Consultation with an applied statistician (Dr Owen Dent, Australian National University) was undertaken for confirmation of the appropriate planning of data collection, design of the research proposal, and methods for analysis of results.

2.14.2 Study Groups

The main study is a case-control study with 48 control and 53 case subjects unless otherwise stated. In the other associated studies undertaken, the number of subjects evaluated will be defined in the particular section.

2.14.2.1 Patients. Males with proven CAD by coronary angiography who were aged less than 50 years at the time of diagnosis and most, but not all, were incident cases.

2.14.2.2 Controls. Males from a population control group who were age, race, socioeconomically and community matched to the study group.

2.14.2.3 Selection of the case-control study groups. The study groups were selected from specific populations and the method of selection is described below. For the preparatory studies, the description of the study groups will be provided within the specific section and appendix. *The limitations of case-control studies are reviewed in Chapter 4. In addition, the problems associated with angiographic studies are discussed in Chapters 8 and 9.*

The same exclusion criteria, as described in Chapter 3, were applied to the case and control groups. The admission criteria for the case group are described in section 4.2.4.

1. Selection of cases. The case group were selected because they had presented with CHD and not because they had undergone cardiac catheterisation. All case subjects, however, had undergone coronary angiography because of clinical indications or because the supervising cardiologist considered their young age at presentation to be an indication.

The case group had clinically stable CHD for approximately 3 months. Exclusion criteria included diabetes mellitus; a stroke, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA) or an acute myocardial infarction (AMI) within 3 months; any significant medical illness, liver and renal disease; those unable to cease aspirin or non-steroidal anti-inflammatory drugs; and any recent surgery or acute illness, in particular, infective or inflammatory disorders.

2. Selection of Controls. The control group were selected by the case group. The case group subjects were requested to ask a friend or work colleague to volunteer for the control group. Except for 4 subjects, all the controls were recruited in this way. The former 4 control group subjects were selected from hospital personnel in the same age and socioeconomic groups as their case group subject who were unable to ascertain a control case. Of these, 3 controls were naive to the laboratory and one was familiar with the laboratory.

Even though the control subjects were personal friends or co-workers of the subjects in the case group, they were required to fit into specific selection and exclusion criteria. Those in the control group were required to have had no cardiac symptoms, normal resting supine ECGs and a normal maximal exercise treadmill test and exercise 12 lead ECG. The exercise test was performed after the control subjects volunteered and a normal result was a selection requirement. They were not selected on the presence or absence of CHD risk factors, although the possibility of a self-selection bias exists.

2.14.3 Variables

Information about each patient and control were documented as outlined (Tables 2.1 and 2.2).

2.14.4 Descriptive Statistics for the Measured Variables

A descriptive assessment of variables was obtained, including histograms plotted for visual inspection of the shape of the distribution, the variability (spread) and to look for unusual outlying measures. Normally distributed data was described by the mean and standard deviation (and variance) for the relevant variables of each group. Further measures of central tendency, namely the median, mode skewness and kurtosis, were determined for variables which may have skewed distributions. This allowed an assessment to be made of the distribution for the variables prior to the univariate, bivariate and multivariate analysis. Those variables measured on a continuous scale were described by:-

1. Histogram (shape and spread of the distribution)
2. Mean and median as measures of central tendency
3. Standard deviation and variance
4. Skewness and kurtosis
5. 95% Confidence limits (indicating the limits for the mean of the population from which the sample was selected).

The above statistics were obtained for all variables in each study group when appropriate, using the Statistical Package for Social Sciences (SPSSX) program.

2.14.5 Measures of Association

Measures of association between variables across groups and between variables within groups were undertaken by the appropriate correlation and regression methods (following the appropriate transformation if the distribution was non-normal and the transformation gave a normal distribution) for continuous variables.

2.14.6 Univariate Analysis

2.14.6.1 Comparisons. The difference of the means between two groups that have a normal distribution was assessed with the Student t-test and paired t-test were appropriate. Testing the differences between the means of the same variable for more than two groups (normal distribution) was by the analysis of variance (ANOVA) programme on the SPSSX.

2.14.6.2 Variable Distributions. If the variables were not normally distributed, then pretest transformation of continuous variables was performed to obtain a normal distribution. If such transformation was unable to change the distribution into a Gaussian distribution, then distribution-free methods to test for differences between means of groups was used. This later choice was not required. The variables with a skewed distribution were all skewed to the left and were transformed to a normal distribution logarithmically. The variables which had a distribution skewed to the left were the LT50 of ADP, adrenaline and collagen, the rate of aggregation with ADP, adrenaline and collagen, the lag phase before the shape change with collagen induced aggregation (LAG), WCC, triglycerides, TXB2 produced in clotted whole blood and beta-thromboglobulin. When they were logarithmically transformed their frequency distribution approximated a normal curve.

2.14.6.3 Categorised Data. The Chi-square test for testing of proportions and association of categorised data within groups was used when appropriate.

2.14.6.4 Regression to the Mean. Regression to the mean refers to the phenomenon that an extreme variable on its first measurement will tend to be closer to the centre of the distribution on later measurements⁵⁵⁰. The possible influence of this potential circumstance is more fully discussed in section 9.4.3 when within person variability is reviewed as a potential limiting factor for the study.

2.14.7 Bivariate Analysis

2.14.7.1 Linear Associations. Bivariate scatter diagrams are examined for evidence of nonlinear relationships. Pearsons product moment correlation coefficients were reported as the measure of association between variables if there was a linear relation.

2.14.7.2 Comparisons of means. Analysis of variance was used to examine the distinct groups which were qualitatively different with respect to the dependent variable. It provides a method of simultaneous comparison of means in order to decide if a relationship exists between variable(s) within a group and across the separate groups.

2.14.7.3 Variable Distribution. Distribution-free methods can be used for comparison of variables which do not have a normal distribution. More commonly, in this study, linear bivariate regression analysis with appropriate transformations for continuous variables was performed. Logarithmic transformation of the continuous variables resulted in normalisation of the values for the variables indicated in section 2.14.6.2.

2.14.8 Multivariate Analysis

2.14.8.1 Background. The simplest approach to evaluate the relationship of a characteristic to the occurrence of future disease is to classify individuals in regard to the characteristic, and subsequently compare classes by their incidence rates of disease in a prospective study.¹⁶² An adequate, although less satisfactory alternative is to evaluate the relationship(s) in a cross-sectional case-control study, which also may involve classification of some of the continuous variables to be examined into discontinuous intervals. Unfortunately problems arise with classification because most biological characteristics are continuous variables, and classifying into discontinuous intervals results in loss of information and implies that associated risk is also discontinuous. More importantly, several characteristics can be simultaneously involved in influencing the development of disease.

An alternative to the classification of variables to examine for any relationship between a characteristic and disease, is to apply appropriate mathematical models. Regression analysis is one such model, and examines the predictive relationship between continuous numerical variables. The variable considered to be influenced by other factors is the dependent variable, and those potentially exerting that influence are the independent variables. The identification of the appropriate independent

variables is required in advance, along with the dependent variable, so that the number of potential independent variables are known before the analysis. The independent variable, within each group, is free to take on different values, and is also numerical.

In this study, a correlation matrix to assess dependent-independent and dependent-dependent relationships was examined. The number of variables to be examined was limited to those considered essential and with substantial preexisting justification for examination. This restriction is necessary in order to decrease the likelihood of a type I statistical error, and was also preferred for the regression analysis.

2.14.8.2 Regression analysis. Selected clinical and laboratory variables which were significantly correlated with a predefined dependent variable were used in multivariate linear regression analysis. The application of multivariable linear regression analysis allows the determination for each variable an estimate of its independent contribution to the variance of the dependent variable. However, regression analysis does not deal with the problem of simultaneous influence of other variables that may be interrelated.

The logistic regression model can be used to yield a probability of the risk which groups have in relation to the independent variable, knowing their dependent variables. This estimated probability has no greater discriminating power than multiple linear regression, but has the advantage of easier interpretation.

2.14.8.3 Definition. Multivariate linear regression analysis will be used to identify subsets of independent variables that are most useful for predicting the dependent variable. This is achieved by developing an equation that summarises the relationship between a dependent variable and a set of independent variables^{1177,1178}.

2.14.8.4 Procedure for selecting the independent variables to enter the regression model. The procedure for choosing the independent variables will be by stepwise forward and backward selection. First the variable will be chosen by forward selection. The variable which is considered for entry into the statistical model is that with the largest positive or negative correlation with the dependent variable. If the criteria for selection are present, the second variable is then selected based on the highest partial correlation. The first variable is also examined to see whether it should be removed according to the removal criterion. After each step, variables already in the equation are examined for removal.

The entry criteria used will be a p value equal to or less than 0.05 for the correlation coefficient. The removal criterion will be a minimum F value of 2.71 in order to remain in the model, and a maximum probability of F being 0.10 for the variable. Variable selection will end when no more variables meet the entry and removal criterion.

2.14.8.5 Summary statistics. The summary statistics reported will include the partial regression coefficient (B), the standard error of the regression coefficient (SE B), the standardised regression coefficient (Beta) and the coefficient of determination (R_{sq}).

The partial regression coefficients (B) is the coefficient for a particular variable after adjustment for other independent variables in the equation. This coefficient does not give the relative magnitude or a measure of relative importance of the association of the independent variable with the dependent variable after adjustment, unless the units of all the variables entered into the regression equation are the same.

The coefficient of determination (R_{sq}) gives a measure of the goodness of fit for a variable in a linear regression model. The statistic is the square of the correlation coefficient between the observed value of the dependent variable and the predicted value of that value determined from the line of best fit from the scattergram. If all the observations fall on the regression line, $R_{sq}=1$. If there is no linear relationship between the dependent and independent variables, $R_{sq}=0$, that is, there is no linear association. Partitioning the sum of squares of the dependent variable gives the proportion of the variation in the dependent variable "explained" by the model and the independent variable.

2.15 ETHICAL CONSIDERATIONS IN HUMAN EXPERIMENTATION

The Division of Clinical Sciences (John Curtin School of Medical Research, Australian National University) considered the research project described and approved it as consistent with the "Statement on Human Experimentation" adopted by the National Health and Medical Research Council. The Division assumed responsibility for ensuring that the work continued to meet the requirements of that statement. The studies undertaken in this thesis were all approved by the Ethics Committee, Woden Valley Hospital, Woden ACT.

Table 2.1. Patient and control characteristics documented.

1. Age
2. Date of onset of symptoms and diagnosis
3. Date, type and number of myocardial infarction(s)
4. ECG and exercise stress test (controls)
5. Stability of angina
6. Time and type of coronary artery bypass grafting
7. Family history (first degree relatives)
8. Time of diagnosis and treatment of hypertension
9. Tobacco intake
10. Presence of clinical peripheral vascular disease
11. Weight, height, body mass index
12. Skinfold thickness
13. Systolic and diastolic BP (phase I and V)
14. Arcus senilis, xanthomata, xanthelasma
15. Medications
16. Alcohol intake, current past and within 24 hr of blood collection
17. Usual dietary intake - fats, cholesterol and fatty acids
18. Behaviour and psychometric measures from questionnaires
19. Blood glucose level
20. Coronary angiography scores

Table 2.2. Laboratory Measures.

1. **Platelet Function Measures**
 1. Plasma platelet count
 2. Mean platelet volume
 3. Platelet count ratio
 4. Plasma Betathromboglobulin
 5. Plasma Thromboxane B2 (TXB2)
 6. TXB2 produced in clotted whole blood
 7. Platelet aggregation
 8. TXB2 produced in response to agonists adrenaline, ADP and collagen
2. **Lipoproteins**
 1. Total cholesterol (past and present)
 2. Total triglyceride (past and present)
 3. LDL-cholesterol (calculated)
 4. HDL-cholesterol (past and present)
3. **Miscellaneous**
 1. Fibrinogen, coagulation parameters
 2. Glycosylated haemoglobin, blood glucose level
 3. Full blood count parameters, ESR, Electrolytes, liver and renal function, and urate

CHAPTER 3

DEMOGRAPHIC FEATURES AND CORONARY HEART DISEASE RISK FACTORS OF THE STUDY POPULATION

3.1 COMPARISON OF THE MAJOR RISK FACTORS FOR CORONARY HEART DISEASE

3.1.1 Coronary Heart Disease Risk Factors

3.1.1.1 Introduction. The term "risk factor" has a number of connotations, including (1) a statistical *correlate* of CHD, usually emerging from multivariate analysis of longitudinal epidemiological studies; (2) a factor that has been identified as a *cause* of CHD; or (3) a characteristic that *predisposes* that person to CHD.³⁷¹ An extensive medical and biological literature has identified over 270 factors which have an association with CHD.²⁰² These factors have been identified according to epidemiological criteria, mostly with a statistical association without necessarily implicating, and certainly not proving causation. In fact, cause and effect relationships have not been demonstrated for the vast majority of risk factors.^{202,235} The main risk factors, however, do have a possible causal relationship with CHD. They include hypertension, tobacco use, hypercholesterolaemia, reduced HDL cholesterol levels and diabetes.³⁷¹ Increasing age, the male sex, and a family history of CHD in a first degree relative are other important factors, although these are not amenable to change. Additional risk factors which may prove to be causal and correctable include an elevated fibrinogen,^{60,62,372-374} specific behavioural factors,³⁷⁵⁻³⁷⁸ abnormal tissue and blood fatty acid profile,³⁷⁹⁻³⁸² and the combination of upper-body obesity, glucose intolerance, hypertriglyceridaemia and hypertension.³⁸³⁻³⁸⁶

Hypertension, elevated cholesterol and smoking contribute heavily to the risk of developing CHD. However, once CHD or CAD has developed, it was previously thought that the presence of these factors may then not alter the prognosis to any great extent.³⁸⁷⁻³⁸⁹ More recent data, however, suggests that this observation may not be entirely accurate.³⁹⁰ In fact there are different risks for patients having angina, with or without a prior MI.³⁸⁹ The prognosis of the individual with established CAD is considered to be related primarily to the nature of the CHD at the time of the diagnosis, viz the extent and severity of CAD and the ventricular contractile function.^{387,388}

3.1.1.2 Risk Factor Intervention. The synergistic interaction of the major risk factors for the development of CHD^{166,391} has lead to the use of multivariate analysis to quantitatively combine risk factors into a composite risk function to increase their predictive accuracy.^{160,371} This same interaction places emphasis on the need to evaluate any individual risk factor by an appropriate multivariate analysis. This is particularly so if one accepts a proposed hypothesis that all other risk factors are associated with CHD through only one independent and causal factor, such as increased LDL cholesterol.³⁹¹ However, a quantitative reduction in single risk factors, disregarding others, may impede any favourable effects on reducing mortality and morbidity from CHD.³⁹² Improved prognostic outcome by attempting to modify the major correctable risk factors has been recently demonstrated.³⁹³⁻³⁹⁸ Nevertheless, the efficacy for such risk factor intervention has been questioned, particularly with alteration of lipoproteins in primary prevention.^{263,263,265,392,394,399-403}

3.1.1.3 Summary. Clearly, whether in those with or without established CHD, a knowledge of all the major risk factors is required in order to evaluate a potential

new factor. The appropriate use of multivariate analysis when employed as a method to control for the influence of the established factors, necessitates that all the relevant factors are entered into the regression model. Moreover, a complete understanding of the causal risk factors and their interaction is necessary in order to plan appropriate therapeutic or epidemiological intervention. Indeed, a greater understanding of the exact mechanisms relating the CHD risk factors to atherogenesis is mandatory in order to identify precisely those at risk to ensure that any intervention will be appropriate in terms of the cost benefit ratio. The need for such an approach has been highlighted in regard to the lowering of cholesterol for the primary prevention of CHD, considering the concerns about the medical cost benefit ratio.²⁶⁴

In this section, the case and control groups are described and compared with regard to their demographic, clinical and risk factor profile. This is to help clarify for the case and control groups, those variables that are important in distinguishing the two groups; to determine if these distinguishing variables are consistent with those in the literature; and to determine those variables appropriate for use in multivariate analysis, recognising the inherent problems of such methods.⁴⁰⁴ The inter-relationship between the major risk factors within each of the study groups has also been evaluated. Any such relationships with measures of platelet function are discussed in Chapter 7. In addition, the relationship of the traditional risk factors with angiographically defined CAD will be considered in Chapters 5 and 8.

3.1.2 Methods

3.1.2.1 Study populations. The patient population consisted of 53 males with CAD proven by coronary angiography, who were aged less than 50 years at the time of examination. The control group consisted of 48 males from a normal population group who were age, race and community matched to the study group. The control subjects had no cardiac symptoms, normal resting supine ECGs and a normal maximal exercise treadmill test and exercise 12 lead ECG. The control subjects were ascertained via the case volunteers and were required to be of a similar age and from a similar socioeconomic group without known cardiac disease.

Clinical information about each case and control was obtained. The case group was required to have clinically stable CHD for at least 12 weeks. Exclusion criteria included individuals with diabetes, a stroke, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA) or non-cardiac surgery within 3 months, any significant medical illness, liver and renal disease, unable to cease aspirin or NSAIDs, and any recent acute illness, in particular, infective or inflammatory disorders.

Information documented included age, date of onset of symptoms and diagnosis, date, type and number of MI(s), family history (first degree relatives) and family risk factors and time of diagnosis and treatment of hypertension, tobacco intake, time of use and lifetime volume, presence of clinical peripheral vascular disease, systolic and diastolic BP (phase I and V Korotoff sounds), the presence of xanthomata or xanthelasma, the type and amount of medications, and alcohol intake (current, past and within 24 hours of blood collection). Judging from the failure to form TXB₂ in clotted whole blood (see Chapter 7), 3 individuals in the case group were

considered to have taken aspirin, and so were excluded from further analysis of platelet function. The anthropomorphic measurements taken were weight, height, body mass index (BMI), and triceps and skinfold thickness. The description of these measures, the methods involved and their limitations are discussed in Appendix 2.

3.1.2.2 CHD risk factors. The methodology for the lipoprotein measurements is described in the methods section in Chapter 2. The past lipoprotein values are historical levels obtained from the patients or their local medical practitioners and the laboratory methods used are not always known.

Indirect BP measurement was made with a cuff and mercury sphygmomanometer as described in Chapter 2, section 2.11.1. The prior diagnosis of hypertension, the time since diagnosis of hypertension, and the time of treatment was obtained from the patient.

Methods for obtaining the family history of CHD and the use of tobacco are described in Chapter 2. Current use of tobacco in those claiming abstinence was not excluded biochemically by urine analysis.

Other variables measured included fibrinogen, HbA1C, blood glucose level and full blood cell count parameters and the methods are described in Chapter 2. Coagulation parameters, viz activated partial thromboplastin time (APTT), the prothrombin time (PT) and thrombin clotting time (TCT), electrolytes and liver and renal function tests were measured by the Pathology Department of Woden Valley Hospital.

3.1.2.3 Statistical analysis. The simplest approach to evaluate the relationship of a characteristic to the occurrence of future disease is to classify individuals in regard to the characteristic, and subsequently compare classes by their incidence rates of disease in a prospective study.¹⁶² An adequate, although less satisfactory alternative is to evaluate the relationship(s) in a cross-sectional case-control study, which may also involve classification of some of the continuous variables to be examined into discontinuous intervals. Unfortunately problems arise with classification because most biological characteristics are continuous variables, and classifying into discontinuous intervals results in loss of information and implies that associated risk is also discontinuous. More importantly, several characteristics can be simultaneously involved in influencing the development of disease. In this section the major risk factors are evaluated as continuous variables or as categorical variables.

A full description of the methodology for the analysis of the data is described in Chapter 2.

3.1.3 Results

The mean age of the control group and the case group were the same, 43.3+/-0.9 and 44.6+/-0.7 respectively (Table 3.8).

3.1.3.1 Anthropomorphic Measures. The height, weight and BPs were identical in each group. The BMI was slightly but not significantly greater in the case group. The case group had significantly greater scapular skinfold thickness which is a meas-

ure of central body obesity (Table 3.1).

3.1.3.2 Clinical characteristics. In the case group only 9 patients had not had a prior myocardial infarct and 44 patients had at least one acute MI, the majority being anterior and inferior infarcts (Table 3.2). The mean time from a previous infarct was 242 days (median 115, standard deviation 317, minimum 12 weeks and maximum 2.7 years). The majority of cases were on cardiac medications and 9 controls were on medications for hypertension or other disorders (Table 3.3).

3.1.3.3 Family history. Fathers of the subjects in the case group had a significantly greater frequency of CHD (Table 3.4). This difference remained of borderline significance even when the unknown cases in both groups were assumed not to have had CHD (Table 3.4). The number of paternal deaths due to CHD were not sufficiently frequent to obtain any reliable information. There were no differences in the known frequency of the major risk factors between the fathers of subjects in both study groups. However, this information was frequently unknown for hypertension and hyperlipidaemia, making any conclusions invalid.

In the two study groups, no differences were found in maternal risk factors, including the diagnosis of CHD (Table 3.5). However, even though the number of unknown observations appears to be relatively low, the sample number in the two groups impart the possibility of a type II statistical error.

The frequency of hypertension was significantly greater in siblings of the patient group, but there were no significant differences in any of the other major risk factors (Table 3.6). There were more smokers among the siblings of cases, but again the difference was not significant. Interestingly, there were more reported diabetics within the siblings of controls rather than the siblings of the cases (Table 3.6).

3.1.3.4. Lipoprotein variables. Of no surprise was the observation that the case group had significantly higher mean total blood cholesterol and triglyceride levels, and a lower HDL level. The past cholesterol level was much greater in those who had had it recorded. However the overlap in individual values between the groups was large, as it was for all the other lipoprotein values (Table 3.7). The apoprotein B level was greater, and the apoprotein A1 level was lower in the case group compared to the controls (Table 3.7). The cholesterol level was not significantly different for the value from the alternate laboratory, but the HDL and triglyceride levels were (Table 3.7).

3.1.3.5 Tobacco use. There were significantly more previous tobacco users in the case group (Table 3.9). In addition, in the case group smokers in general smoked a greater amount over their lifetime (Table 3.8).

3.1.3.6 Hypertension. More cases than controls were diagnosed as having hypertension, and the number of cases requiring treatment was similarly greater. Both these differences did not, however, attain statistical significance with the study sample number (Table 3.9). The highest previously recorded systolic and diastolic BP recalled by the subjects were significantly greater in the case population (Table 3.8). The BPs at the time of examination were not significantly different, which was

presumably related to adequate treatment, or possibly due to previous MIs (Table 3.8).

3.1.3.7 Blood glucose levels. The blood glucose level and HbA1C were greater in the case group even though overt diabetics were excluded. There were no outliers in the frequency distribution and the variables were normally distributed (Table 3.10).

3.1.3.8 Fibrinogen. The fibrinogen level in the case group was greater than in the controls and in these two study groups it was normally distributed (Table 3.8).

3.1.3.9 Alcohol use. A slightly greater percentage of controls continued to use alcohol at the time of the interview (Table 3.9). Alcohol use is evaluated in more detail in Chapter 5.

3.1.3.10 Haematological measures. The case group had a significantly higher Hb and a lower MCV than the controls. The Hc, WCC, PT and APTT were not significantly different between the two groups (Table 3.10).

3.1.4 Discussion

3.1.4.1 Sex as a risk factor. In populations in which CHD is a major health problem, the incidence of CHD is markedly lower in premenopausal women than in men, although there is a convergence after menopause. By the 8th decade the incidence is almost equal in the two sexes.¹⁹⁶ National mortality data in some Western countries suggests that the decreasing sex difference in CHD after 50 years of age is due to a declining rate of increase in men rather than an acceleration in women.¹⁹⁶ This data suggests that factors other than age per se are more important in the development of CHD, even though atherosclerosis continues to accumulate with age. The cause for the difference may be due to a mortality bias from premature deaths.

The relationship of risk factors to CHD also appears to be different in females. Women have much lower rates of CHD than men at the same values of cholesterol,^{198,405} menopause has an unfavourable effect on lipids,⁴⁰⁶ diabetes mellitus seems to be a greater risk factor for females than males^{407,408} and the genetic component to the liability to cardiac death may be greater in women.⁴⁰⁹ Women who die from CHD appear to have a similar relative risk but lower absolute and attributable risk for smoking, diastolic BP and social class than men.⁴⁰⁵ However, the overall impact of CHD risk factors in women is poorly understood, the reason(s) for the different natural history are unknown and the pattern of risk associated with factors pertaining solely to women is even less well understood. Indeed, the evaluation of CHD risk factors in longitudinal epidemiological studies indicate that some other factors protect women against CHD, such as natural oestrogen.⁴⁰⁵

Considering these and other concerns, it was considered prudent to again endeavour to make the study population as homogeneous as possible and initially exclude young women with CHD. The mothers of the two study groups had no significant differences in the incidence of CHD or CHD risk factors, although this may simply be a type II statistical error.

3.1.4.2 Age as a risk factor. Since aging itself is a well recognised and powerful risk factor for the development of CHD,⁴¹⁰ a group of males with premature CHD and a narrow age range and an age matched control group were selected to exclude the influence of age on atherosclerosis and risk factors. In addition, choosing a young group would presumably reduce the confounding effect of the longer term influence of the major risk factors which themselves increase with age.^{161,202}

3.1.4.3 Family history. A number of previous case-control studies have evaluated the familial occurrence of CHD as a primary objective.^{177-180,411-413} An increased risk of CHD in relatives of individuals with the disorder may in part be due to genetic factors.^{177,411,413} This relationship was found to be highest when parental death from the disease occurred prematurely,^{177,411} particularly in males who have both parents dying of CHD.⁴¹¹ Similar patterns were not detectable in older age groups.⁴¹¹

The familial component for premature CHD is also related to the aggregation of risk factors in families.^{179,411} The magnitude of this contribution is controversial, with some studies demonstrating this as the major or total cause of the familial pattern.^{178,414,415} The relative importance of the individual risk factors is similarly variable.^{179,221,414,415} The inherent problems in these studies is that a primary risk factor may reflect a genetic tendency to the major risk factors, or a shared environmental influence common within families.⁴¹⁵ There may even be a familial aggregation for an increased susceptibility to the effects of the major risk factors.⁴¹⁵ Nevertheless, support for case-control studies demonstrating a separate association of an independent genetic contribution is impressive.^{170,188,416} In particular, prospective studies endorse the possibility of heritable factors as important determinants of cardiovascular mortality mainly in younger men,^{171,181,188,417} and that CHD occurs partly as a familial disorder, particularly evident in males with premature CHD.^{171,181,188,417} One large prospective study indicates that a history of AMI in either parent is associated with an increased risk of CAD among men independent of diet or established risk factors.⁴¹⁷ Indeed, the weight of evidence indicates that the distinct familial contribution to the major risk factors fails to account for all of the inherited component^{188,416} and that other familial risk factors remain to be found.⁴¹⁶

The primary aim of this present study was not to examine familial aggregation of CHD. The study number is too small to evaluate the independence of such an association by adjusting for other CHD risk factors. Further, a positive recall bias in those having an AMI cannot be controlled for without independent validation of the subjects personal observations and recollections.

The significantly increased incidence of paternal CHD in this study is concordant with the literature. However, this observation, and that for the siblings and the maternal history, cannot be interpreted as a positive or negative finding in view of the limitations discussed above. However, the selection of young males as the study population was in part motivated by the evidence that premature CHD in males does have a significant and independent familial aggregation,^{181,188,416,418} which is unexplained. The selection of such a group may therefore increase the possibility of detecting such unidentified risk factors by reducing the "noise" from interactive confounding factors and heterogeneous study populations.

3.1.4.4 Lipoproteins. A relationship between increased platelet aggregability and platelet reactivity with dyslipoproteinaemias has been well established,^{126,128,419-426} highlighting the importance of carefully documenting the lipoprotein profile of the study group. This aspect is discussed in Chapter 5.

3.1.4.5 Hypertension. It also appears possible that a relationship between BP and platelet aggregation exists.¹⁴⁵⁻¹⁴⁷ The fact that hypertension was treated, particularly in the case group, many of whom were on vasoactive medications for other reasons, may be a potentially important confounding factor which cannot be effectively controlled for in this cross-sectional study. Appropriate interpretation of the results must be made given this potential major bias.

3.1.4.6 Blood glucose. Raised plasma adrenaline and hyperglycaemia after MI may activate platelets, and this could contribute to a poor outcome in such patients.⁷⁵ A relationship between platelet function and abnormal glucose metabolism is well established, although not fully clarified, and illustrates the necessity for excluding diabetic individuals. Since abnormal glucose homeostasis may play a role in the pathogenesis of CHD, even in non-diabetics, an evaluation of relationships with glucose haemostasis has been undertaken. This aspect is discussed in Appendix 2.

3.1.4.7 Tobacco use. Previous studies have suggested that platelets may be activated during chronic cigarette smoking^{57,427,428} and that the metabolism of platelets is altered. For example, increased production of thromboxane may be a direct effect of smoking.^{428,429} Furthermore, there appears to be an acute effect of cigarette smoking on *in vitro*^{430,122} and *in vivo* platelet function, although this is controversial.^{431,432} Smoking cessation lessens the risk of death or MI in older and younger persons with CAD.⁴³³ Indeed, the increased risk of a first MI among cigarette smokers declines soon after cessation, both for men and women, and is largely dissipated within two or three years.⁴³⁴ In fact, the risk of smoking would appear to be associated only with current smoking, and the duration of the smoking habit is less important.⁴³⁵ Moreover, non-atherogenic mechanisms may be important in the aetiology of MI among women who are cigarette smokers.⁴³⁶ A rapid regression in risk of CHD within the first year of quitting smoking is difficult to explain solely on the basis of vascular disease,⁴²⁹ and may be related to a reversal in abnormalities of platelet and haemostatic function.⁴²⁹

In this study, very few controls or cases were current tobacco users. Therefore, in light of the foregoing discussion, there is a high probability that the study may suffer from the confounding influence resulting from the cessation of smoking. On the other hand, the fact that *the majority did not use tobacco at the time of the evaluation, enables the detection of any abnormal platelet function in the case group independent of acute tobacco use.*

3.1.4.8 Cardiac medications. Beta-blockers without intrinsic sympathomimetic activity are associated with an increase in the triglyceride level and a decrease in the HDL cholesterol level.³⁸⁹ Despite these unfavourable changes, after AMI, there is a reduction in risk associated with the use of beta-blockers.³⁸⁹ Thiazides also influence lipids, resulting in an increase in total cholesterol.⁴³⁷ The longterm outcome of these changes are unknown.⁴³⁸ More importantly, a number of cardiac drugs influence

platelet function *in vitro* and may do so *in vivo* as well. Therefore, a separate evaluation of the influence of the more common types of cardiac medications was undertaken as a preliminary evaluation. The results are presented and discussed in Chapter 4.

3.1.4.9 Conclusion. This section details important comparisons between the two groups in this case-control study, in particular with regards to the major risk factors for CHD. It is clear that the differences between the two groups are consistent with those found in the literature and clinical practice. Important potential confounding factors apparent from these results are the differences in the lipoproteins, glucose homeostasis, plasma fibrinogen levels, possibly previous tobacco consumption and BP levels. Furthermore, methodological bias may arise from the greater use of cardiac drugs in the case group. These issues of methodological bias and the presence of confounding factors are addressed in Chapters 4 and 8.

3.2 MARITAL STATUS, EDUCATION AND EMPLOYMENT

3.2.1 The Necessity of Matching the Case and Control Groups for Socioeconomic Status

3.2.1.1 Introduction. The factors attempted to be matched for the case and control groups were age, sex and socioeconomic status. Therefore, to ensure this pre-evaluation matching was successful, an analysis of the education level achieved and marital status was undertaken. In addition, the reasons as to the necessity of this approach will be discussed.

3.2.1.2 Socioeconomic Status as a risk factor for CHD. An increased overall mortality in the lower socioeconomic groups of Western countries is well documented.⁴³⁹⁻⁴⁴² Indeed, the potential contribution of the risk for the lower socioeconomic groups to overall mortality is considerable.⁴⁴² Furthermore, an association between CHD and low social and economic class has been consistently demonstrated.⁴⁴⁰⁻⁴⁴⁸ These numerous reports have originated from a number of different countries and from varied investigators using diverse methodologies, factors which enhance the validity of the observations. Not only do lower socioeconomic groups have an increased incidence of CHD, but the symptoms of the disease and the functional impairment is greater. Symptoms of cardiovascular disease, impaired working capacity, functional disability, anxiety and sleeping disorders have been reported among unskilled workers in a number of different surveys.⁴⁴⁹

3.2.1.3 Shift Work. An association between shift work and increased risk for the development of CHD has been well described.^{450,451} This particularly applies to the first two decades of shift work, with a decrease in the association subsequently probably being due to positive selection taking place in the study group.⁴⁵² In addition, the association was independent of age and smoking habits.⁴⁵² The more recent observations⁴⁵⁰⁻⁴⁵² have been consistent, although earlier studies found either no relationship between shift work and cardiovascular disease,^{453,454} or a similar but not significant direction to the association.^{455,456} However, the earlier studies had a number of design faults that could lead to erroneous results which the contemporary

studies attempt to rectify, improving the reliability of the observation.⁴⁵²

One possible mechanism that may enhance the risk for shift workers is the higher incidence of blood lipid abnormalities.^{452,453,457,458} The abnormalities appear to be independent of smoking habits, obesity, dietary factors and leisure activity.⁴⁵⁸ Shift workers have been reported to have an increased consumption of tobacco,⁴⁵² although there is a risk independent and in addition to this increased consumption.⁴⁵²

3.2.1.4 Risk factors for CHD and socioeconomic group. Socioeconomic and social support variables have clearly been related to CHD risk factors.^{459,460} Life-style and an uneven distribution of established risk factors for CHD may be the most obvious explanations for the differences in CHD between social class.⁴⁴² The increased incidence of hypertension in the lower socioeconomic groups has been uniformly reported from a number of diverse Western populations,⁴⁴²⁻⁴⁴⁵ including Australia.⁴⁴⁵ Differences between populations with regard to the other major risk factors have been noted. The frequency of tobacco use and the levels of blood cholesterol are increased in lower socioeconomic groups in Great Britain⁴⁴³ but not Sweden.⁴⁴² Tobacco use and tobacco-related illness, including CHD, is greater in lower socioeconomic groups in Australia.⁴⁶¹ Blood lipid abnormalities are not consistently related to educational level, except possibly for HDL and triglyceride levels in women taking a more beneficial direction with education level.⁴⁶² A height difference, lower socioeconomic groups being on the average shorter than higher socioeconomic groups^{442,443} may reflect differences in genetic constitution as well as nutritional factors during childhood.⁴⁴²

3.2.1.5 Geographic Variations. Pronounced variations in cardiovascular mortality have been identified in a number of Western countries.⁴⁶³ The British Regional Heart Study indicates that such a variation in CHD rate among middle aged men cannot be directly and solely explained by their genetic inheritance, or by perinatal and postnatal dietary differences.⁴⁶³ Indeed, prevalence studies between population groups within the United Kingdom demonstrate differences in mortality between areas, in particular lower mortality in areas where employed men constituted the patient population.⁴⁶⁴ Differences in community or medical services, smoking habits or unemployment rates appeared to offer an explanation for the variations in CHD mortality.⁴⁶⁴

3.2.2 Results

The level of education achieved by the individuals in the case and control groups is listed in Table 3.11. A contingency table with a 9 by 2 matrix containing the number of subjects that were classified as part of each column and row, with each subject appearing in only one cell, was employed for the analysis. The Chi-square statistic with Yates correction was used to quantitate any association between the columns and rows, that is whether being in either the case or control group influenced the education level. There was no measurable difference between the study groups and the level of education attained (Chi-square statistic=8.2899, p=0.4057).

Dividing the groups into those achieving some tertiary level of education or matriculation to a University, both of which correlate with socioeconomic status, provided a

2 by 2 contingency table for the application of the Chi-square statistic with the Fisher exact test (Table 12). Again, there was no significant difference between the groups (Chi-square statistic=1.2681, Fisher exact test 2-tailed $p=0.2316$, with an Odds ratio=0.585).

The overall marital status of the two groups was also not significantly different (Table 13).

3.2.3 Discussion

There is no major difference in the socioeconomic status as measured by marital status and education level. By using community matched controls, mainly being friends of the case group, it is probable that the potential bias associated with the socioeconomic status of the two groups has been prevented or at least reduced. Nevertheless, the socioeconomic difference may be due to measurable risk factors already taken into account within the study design.

3.2.3.1 Vegetarians, Seventh Day Adventists and Coronary Heart Disease. Mortality from CHD appears to be lower in vegetarians compared to non-vegetarians, particularly among men.^{465,466} The assumptions that vegetarianism confers some protection against CHD appears valid although the exact reason has not been determined.⁴⁶⁵⁻⁴⁶⁷ Predictably vegetarians have lower serum cholesterol and body mass index (Weight/height squared),^{465,468} although it remains unclear whether the low cholesterol levels of vegetarians are due to meat abstention and/or the high intake of vegetables.⁴⁶⁶

The epidemiological relationships to the incidence of CHD and CHD risk factors have been extensively studied in Seventh-Day Adventists.⁴⁶⁵ Adventist men have lower rates of CHD mortality, although the information regarding Adventist women is conflicting.⁴⁶⁵ This religious group have lower rates of cholesterol and BP, but also lower levels of HDL.⁴⁶⁵ Available information indicates that the lower risk for CHD in Adventist men is probably related to their dietary habits, non-smoking status, greater social support and possibly their better exercise habits.⁴⁶⁵

However, the low BP and cholesterol levels found in Adventists may be due to a selection bias.⁴⁶⁹ In addition, the effect of psychosocial factors within this population has been difficult to determine. Examination of the data from the Tromso Heart Study has further underlined the difficulty in identifying specific aspects of Adventists lifestyle responsible for the mortality differential.⁴⁶⁹ Review of the overall information available suggests that the lower levels of BP and cholesterol in religiously active Adventists is not due to a selection bias or religious factors other than those influencing life-style.⁴⁶⁹

There were no differences in the frequency of vegetarians in the two study groups as documented in Chapter 5. However, the religious status of the two groups was not documented, although a significant difference is unlikely. This lack of documentation is unlikely to lead to a bias within the study, especially given the probability that the difference in CHD incidence in religious groups compared to the general popula-

tion is due to differences in risk factors. Nevertheless, the possibility of a difference exists and could lead to a bias which confounds the results.

3.2.3.2 Conclusions. The lower socioeconomic groups in Western countries are more affected by the incidence of CHD, the symptoms and disability arising from the disease and the risk factors associated with CHD. Further, specific occupational characteristics prevalent in the lower socioeconomic groups have in themselves a risk association with CHD, both the type of work performed and when it is undertaken. The existence of possible confounding factors related to the life-style of the socially and economically disadvantaged, particularly in relation to dietary factors, cannot be dismissed. Seventh-Day Adventists provide a relatively homogeneous population with specific life-style characteristic which appear to carry a low CHD risk, as do vegetarians. These studies implicate dietary factors and smoking habits. The differences between the socioeconomic groups are qualitatively similar to those demonstrated between Adventists and non-Adventists, and vegetarian groups and non-vegetarians. Specifically, Seventh-Day Adventists, vegetarians, higher socioeconomic groups and non-shift workers have lower intakes of tobacco, saturated fat in their diet, lower blood cholesterol and lower BP than non-Adventists, non-vegetarians, lower socioeconomic groups and shift workers. There are clear indications that specific life-style characteristic contribute and provide a potential area for intervention in lower socioeconomic groups, both from a primary prevention and secondary prevention perspective.

Given the similarity in the education level and marital status of the two groups, and the possibility that the documented differences in CHD risk between socioeconomic groups is related to risk factor and dietary differences, it is unlikely that any major bias will have occurred in the case-control study described in this Thesis.

Table 3.1. Anthropomorphic measures.

	Controls Mean+/-SE	Cases Mean+/-SE	95% CI
BMI (kg/m ²)	25.7+/-5.0	26.6+/-5.0	-5.01 to 23.07, p=0.2072
Triceps (cm)	12.3+/-1.0	12.5+/-0.7	NS
Scapula (cm)	13.8+/-0.7	17.1+/-0.9	0.96 to 5.64, p=0.0064
Height (cm)	176.0+/-1.2	175.0+/-1.1	NS
Weight (kg)	80.0+/-1.7	80.8+/-1.5	NS

BMI=body mass index, triceps=triceps skinfold thickness, scapular=scapular skinfold thickness, CI=confidence interval of the mean difference, NS=no significant difference.

Table 3.2. Myocardial infarcts in the case group.

	Number of MI	Position of MI
Nil MI	9	Anterior 18
Single MI	40	Inferior 21
Two MI	3	Lateral 7
Three MI	1	Indeterminant 3

Numbers refer to the number of subjects.

MI=myocardial infarction.

Table 3.3. Medications used by subjects in the two groups.

	Controls	Cases
Beta-blockers	0	24
Calcium blockers	3	22
Nitrates	0	13
Vasodilators	2	0
Antiarrhythmics	0	3
Digoxin	0	2
Diuretics	1	4
Theophylline	1	0
Others	5	4

Numbers refer to the number of subjects.

Table 3.4. Father's medical history relevant to CHD.

	Controls	Cases	
Diagnosis of CHD:			
Present	11	21	Chi-S = 8.0816 p = 0.0176
Absent	36	26	
Unknown	1	6	
Present	11	21	Fishers' Test p = 0.0492 OR = 2.643
Absent	36	26	
(disregarding missing values)			
Present	11	21	Fishers' Test p = 0.0886 OR = 2.207
Absent	37	32	
(assuming missing values negative)			
Death due to CHD:			
Present	7	13	Chi-S = 1.1189 p = 0.5712
Absent	14	16	
Unknown	1	3	
Present	7	13	Fishers' Test p = 0.3005 OR = 0.615
Absent	14	16	
(disregarding missing values)			
Diagnosis of Hypertension:			
Present	8	8	NS
Absent	34	30	
Unknown	6	15	
Tobacco Use:			
Present	28	34	NS
Absent	19	17	
Unknown	1	2	
Diagnosis of Diabetes:			
Present	1	4	NS
Absent	46	46	
Unknown	1	3	
Diagnosis of Hyperlipidaemia:			
Present	0	0	NS
Absent	23	17	
Unknown	25	36	

Chi-S=Chi square statistic, OR=odds ratio, NS=no significant difference.

Table 3.5. Mother's medical history relevant to CHD.

	<u>Controls</u>	<u>Cases</u>	
Diagnosis of CHD:			
Present	11	11	
Absent	36	41	NS
Unknown	1	1	
Death due to CHD:			
Present	7	8	
Absent	8	9	NS
Unknown	1	1	
Diagnosis of Hypertension:			
Present	11	17	
Absent	32	31	NS
Unknown	5	5	
Tobacco Use:			
Present	11	18	
Absent	36	34	NS
Unknown	1	1	
Diagnosis of Diabetes:			
Present	4	6	
Absent	43	45	NS
Unknown	1	2	
Diagnosis of Hyperlipidaemia:			
Present	5	5	
Absent	19	20	NS
Unknown	24	28	

Table 3.6. Siblings' medical history relevant to CHD.

	Controls	Cases	
Diagnosis CHD in Brother:			
Present	2	4	
Absent	45	47	NS
Unknown	1	2	
Diagnosis CHD in Sister:			
Present	1	0	
Absent	46	52	NS
Unknown	1	1	
Death due to CHD:			
Present	0	0	
Absent	48	53	NS
Diagnosis of Hypertension:			
Present	5	18	Chi-S = 8.1345
Absent	38	32	p = 0.0171
Unknown	5	3	
Present	5	18	Fishers' Test
Absent	38	32	p = 0.0253
(disregarding unknowns)			OR = 0.234
Tobacco Use:			
Present	11	18	
Absent	36	34	NS
Unknown	1	1	
Diagnosis of Diabetes:			
Present	7	1	Fishers' Test
Absent	40	51	p = 0.0253
Unknown	1	1	OR = 8.925
Diagnosis of Hyperlipidaemia:			
Present	7	3	
Absent	26	34	NS
Unknown	15	16	

See Table 4 for abbreviations.

Table 3.7. Lipoprotein measures in the two study groups.

Variable	Controls Mean+/-SE	Cases Mean+/-SE	95% CI Mean Diff
Past chol (mmol/l)	5.7+/-0.3 (n=15)	6.1+/-0.3 (n=27)	-0.53 to 1.33, p=0.39
Present Chol (mmol/l)	4.9+/-0.1	5.2+/-0.1	0.02 to 0.58, p=0.037 *
Triglycerides (mmol/l)	1.1+/-0.1	1.9+/-0.2	0.15 to 0.31, p<0.0001 *
HDL chol (mmol/l)	1.00+/-0.04	0.86+/-0.03	-0.24 to -0.04, p=0.0057 *
Chol/HDL	5.2+/-0.2	6.3+/-0.2	0.54 to 1.66, p=0.0002 *
Apoprotein A (mmol/l)	1.50+/-0.07 (n=37)	1.21+/-0.04 (n=44)	0.14 to 0.44, p=0.0003 *
Apoprotein B (mmol/l)	0.82+/-0.04 (n=37)	0.95+/-0.03 (n=44)	0.20 to 0.24, p=0.0098 *
ApoA/ApoB	2.00+/-0.15 (n=37)	1.31+/-0.06 (n=44)	0.39 to 0.99, p<0.0001 *
Alternate Chol (mmol/l)	5.45+/-0.13 (n=46)	5.64+/-0.15 (n=52)	-0.59 to 0.21, p=0.3469
Alternate Trig (mmol/l)	1.11+/-0.12 (n=46)	2.24+/-0.24 (n=52)	-1.73 to -0.53, p=0.0003 *
Alternate HDL (mmol/l)	1.29+/-0.07 (n=46)	0.95+/-0.03 (n=52)	0.20 to 0.48, p<0.0001 *

Alternate=measurement performed in an alternative service laboratory using plasma, chol=cholesterol, trig=triglyceride, HDL=HDL cholesterol, CI=confidence interval of the mean difference. Values are expressed as mean+/-SE.

Table 3.8. Other risk factors for CHD.

	Controls Mean+/-SE	Cases Mean+/-SE	95% CI Mean Diff
Age (years)	43.3+/-0.9	44.6+/-0.7	NS
Fibrinogen (mg/100ml)	230 +/-8	275 +/-10	18.61 to 71.39, p=0.0011 *
Packyears (Years)	22 +/-4.7 (n=19)	37 +/-3.4 (n=34)	-0.09 to 0.27, p=0.33
Previous SBP (mmHg)	128 +/-3 (n=36)	144 +/-5 (n=35)	4.44 to 27.56, p=0.007 *
Previous DBP (mmHg)	83 +/-3 (n=35)	93 +/-3 (n=37)	2.52 to 17.48, p=0.0095 *
Current SBP (mmHg)	125 +/-2.2	123 +/-1.6	NS
Current DBP (mmHg)	80 +/-1.6	79 +/-1.9	NS
BSL (mmol/l)	4.9+/-0.08	5.2+/-0.10	0.04 to 0.56, p=0.0222 *
HbA1C	7.3+/-0.1	7.8+/-0.2	0.05 to 0.95, p=0.0307 *
Creatinine umol/l	99 +/-2	97 +/-3	NS

Packyears=equivalent number of years of smoking at 20 cigarettes per day, SBP=systolic BP, DBP=diastolic BP, previous=highest previous BP recalled by the subject, current=at the time of the subject's interview for the study, BSL=blood glucose level, HbA1C=glycosylated Hb level, NS=no significant difference, * = significant difference, CI=confidence interval of the mean difference.

Table 3.9. Other risk factors for CHD.

	Controls	Cases	Fishers' Exact Test
Current smokers	7	4	p=0.34 OR=2.1
Previous smokers	19 (40%)	34 (64%) *	p=0.04 OR=0.4
Known hypertension	12 (25%)	21 (40%)	p=0.14 OR=0.5
Treated hypertension	9 (19%)	19 (34%)	p=0.08 OR=0.4
Previous Alcohol use	44 (92%)	44 (83%)	p=0.13 OR=3.0

*=significant difference between groups, OR=odds ratio.

Table 3.10. Haematological values in the two study groups.

	Controls Mean+/-SE	Cases Mean+/-SE	95% CI Mean Diff
Hb (gm/dl)	14.8+/-0.01	15.1+/-0.1	0.09 to 0.51, p=0.0055
Hc (%)	43.2+/-0.4	43.6+/-0.3	-0.58 to 1.38, p=0.4202
MCV	90.4+/-0.6	89.0+/-0.5	-2.55 to -0.25, p=0.0173
RDW	12.9+/-0.08	12.8+/-0.09	NS
WCC	6196 +/-255	6698 +/-233	NS
PT	13.3+/-0.1	13.6+/-0.4	NS
APTT	24.4+/-0.4	24.2+/-0.8	NS

Hb=haemoglobin, Haematocrit=Hc, MCV=mean red cell volume, WCC=white cell count, PT=prothrombin time, APTT=activated partial thromboplastin time, NS=no significant difference, CI=confidence interval of the mean difference.

Table 3.11. Education level achieved.

<u>Education Level</u>	<u>Case Group</u>	<u>Control Group</u>
Primary school alone	3	1
Junior high school alone	4	3
Junior high school certificate	11	8
Senior high school alone	4	-
Senior high school certificate	6	7
Matriculation	3	5
Tertiary education not graduated	8	9
Graduated tertiary education	12	10
Post-graduate qualifications	2	5

Table 3.12. Case and control group divided into those with and without some tertiary education.

<u>Tertiary Education or matriculation</u>	<u>Case Group</u>	<u>Control Group</u>
Yes	25	29
No	28	19

Table 3.13. Marital Status

<u>Marital Status</u>	<u>Case Group</u>	<u>Control Group</u>
Never married	4 (7.5%)	2 (4.2%)
Married	41 (77.4%)	40 (83.3%)
Separated	5 (9.4%)	-
Divorced	3 (5.7%)	5 (10.4%)
Widowed	-	1 (2.1%)

CHAPTER 4

EVALUATION OF POTENTIAL AREAS OF MAJOR METHODOLOGICAL BIAS

4.1 CASE-CONTROL STUDIES

In this section, the limitations of the case-control method are outlined. The following discussion illustrates that a "simple" case-control study requires careful preparatory evaluation because of the number of potential confounding factors. These include confounding factors arising from methodological bias which may degrade the final information and invalidate the conclusions. The case-control method is cross-sectional and begins after several innate biases have already occurred, some being uncorrectable. Further attempts to correct some of the bias may augment existing bias or create an "investigative bias".^{370,470,471} Nevertheless, in the planning of a case-control study, an analysis of major potential areas of bias arising from the design of the study should be undertaken.^{472,471}

4.1.1 Definition of a Case-Control Study

A case-control study is an example of a cross-sectional study, the purpose of which is not only to give a qualitative description of disease in a population at a given time, but to also allow quantitative assessment of characteristics within the population in relation to the disease.^{370,470,471,473} In simple terms, a case-control study compares people with and those without disease. The comparison is to see if they differ in their exposure to a suspected cause or contributing influence for the disease. In doing so, cases are assumed to be representative of the population with the disease. Similarly, the controls are assumed to be representative of the population in which the disease cases arose, but who do not have the disease in question at the time. The comparison group is used to support or refute the inference of an association for any particular factor. The presence of any confounding factors influencing the relationship between the variable in question and the outcome, needs to be adequately matched. Comparability of the groups can be improved by correct matching of such potentially confounding factors which already are known to influence the outcome. Alternatively, their effects can be allowed for in the appropriate analysis, provided they are recognised prior to the commencement of a cross-sectional study and are adequately documented.^{471,472}

4.1.2 Rational for the Use of the Case-Control Method

Experimental and observational studies, in an attempt to elucidate some of the aetiological factors of CHD and atherosclerosis, have demonstrated an association between the acute symptomatic CAD syndromes and measures of increased platelet activity (Chapter 1). Evidence supporting a similar association between increased platelet reactivity with stable CHD and/or atherosclerosis, is more tenuous. An established way to evaluate such a possibility is firstly to determine whether the characteristics in question occur more frequently or are greater in persons with the disease. Then, prospectively determine whether persons with the attribute(s) develop the disease more frequently than those without. Therefore, an initial evaluation of an hypothesis can be by the cross-sectional case-control study in which the characteristics being examined are identified in both the cases and controls at the time of the data collection. The proportion of cases possessing the variables of interest is compared to the corresponding proportion in a control group. A higher prevalence of the study factors in cases compared with the controls indicates a possible association

between these factors and the disease.

The question of causal inference between the study variables and the disease would need to be subsequently evaluated. This is particularly important when the study factors are physiological variables, which are more likely to be influenced by the disease itself, or by treatment.

Experimentation and determination of biological mechanisms provide the most direct evidence for causal relationships. Although such evidence is usually not available from case-control studies, the method can be used in epidemiological investigations to detect potential causal relationships between the variables and disease. Indeed, such methods are often the most appropriate, or even only methods, to investigate phenomena occurring in free-living intact humans. They may provide sufficient knowledge to justify more extensive evaluation or for the confirmation of existing information.

4.1.3 Definition of a Case

A case defines a group of individuals who have a disease which, as far as possible, is a homogeneous aetiological entity. Existing knowledge, whether manifestational, epidemiological or experimental, should be used to define such a homogeneous condition.⁴⁷³ Each eligible case in the target population should have an equal chance of inclusion in the study, irrespective of exposure to the study variable. In a population based case-control study, cases have the study disease occurring within a defined geographical area during a specified period of time. Ideally the case group should comprise a representative random sample of that population with the disease.^{370,470,471}

4.1.4 Definition of a Control Group

The control group is used to compare the presence of an abnormality, or evidence of exposure to the variable(s) in question in the cases, with that in individuals who are free of the study disease. In fact, a control provides an estimate of exposure rate to the characteristics being studied among individuals free of the disease. This would be the expected rate in the cases if there were no association between the study disease and the variables. Accordingly, the control individuals should be not only free of the disease but similar to the cases with regards to the likelihood of exposure to the variables in question during the time period of the risk for the development of disease. The controls should also have an equal chance of selection, irrespective of the presence or absence of study variables. As with cases, the control group can be hospital-based or population based. Any factors which are already known to be associated with the disease must be taken into account. Even if an association is only strongly suspected, such a possible relationship should be matched or adjusted for in the analysis. It is also possible, even in random selection, that some factors may not be evenly distributed in the control population, particularly with smaller sample sizes.^{370,470,471}

4.2 LIMITATIONS OF CASE-CONTROL STUDIES

4.2.1 Disadvantages of Case-Control Studies

As with all cross-sectional studies, by definition, it is only possible to estimate the prevalence of disease, and not the incidence. The prevalence depends on a balance between the rate at which new cases arise and the rate at which established cases are removed from the population. The removal rate is dependent on recovery rate, mortality rate and factors such as migration. These and other limitations affecting cross-sectional studies also apply to case-control studies and, therefore, will affect the reliability of the analysis and, as a consequence, the conclusions.

An understanding of the possible confounding factors of the case-control methodology is necessary, some of the more important being outlined below. Firstly, there is the potential for selective removal, such as the systematic omission of less healthy individuals leading to under assessment. Those subjects with the disease who are not characterised because of death may have different study features as compared to the survivors. Secondly, characteristics observed at the time of the study may not be the same as those present during the formation of the disease, the characteristics under study being altered by treatment or the illness. These difficulties with case-control studies arise from the fact that the methods involved proceed from the result to the cause and effect relationships, thus following a paradigm which is retrospective.⁴⁷¹ Thirdly, the cases and/or controls selected may not be representative of the disease in the population, or of the population as defined. This uncertainty in ascertainment presents the problem of selection bias. The assumption of complete representation would only be correct if all patients with the disease received attention or were detected, and the prevalence of the variables under study was the same in the control group as in the general population. Finally, it is not always possible to ensure that known confounding factors or study variables are equally distributed between groups. Some factor which is unidentified by the investigator may be responsible both for the exposure and the disease, being the outcome of the exposure. Blindness to outcome can be difficult to achieve, allowing observer bias to enter the comparisons. The inability of the method to assess the incidence of the disease, and the fact that it involves a late examination of those affected earlier, means those with fatal or short episodes will be missed. Mild or asymptomatic cases, and those in which evidence of exposure disappears or is modified by disease, may also be missed.

These examples of the disadvantages inherent in the case-control methodology illustrate the necessity to consider and identify the various sites of innate bias within the study. Also the possible, indeed one should assume probable, confounding factors which may distort the study results need to be delineated as far as is possible, prior to commencement of a study in order to make appropriate changes in the method and protocol.^{370,470,471,473,473} The areas of potentially major bias are discussed in detail in the following sections.

4.2.2 Bias in the Case-Control Study

In a case-control study, the compared groups need to be similar in all respects except for the outcome being investigated. This is to ensure that any difference found in the

pertinent study characteristics is indeed a true finding and not distorted by bias. The principle of "ceteris paribus" can then be applied.⁴⁷¹ Therefore, correct matching for major variables which may influence the variables being investigated must be incorporated into the design. Post-stratification can be used for less important confounding variables, or for those factors with uncertain effect.^{370,470,471} To ensure the correct study design that takes appropriate care to avoid and to define any confounding factors, it is necessary to systematically examine the design for each potential methodological bias.

4.2.2.1 Susceptibility bias. Susceptibility bias occurs at the selection of groups for comparison, and refers to the bias introduced into a study if the baseline groups have different prognostic expectations for the outcome of interest. Subjects in one group may have negative prognostic features, or alternatively, the "healthy worker effect", both of which can provide major disadvantages or advantages for the outcome. This form of bias arises from problems such as self selection, suitable compliance to the study, vulnerability to a manoeuvre or attribute, and even patterns of referral or migration. Ideally, randomisation of the selection of subjects reduces the likelihood of differences in allocation producing disparate groups.

In case-control studies, susceptibility bias can be reduced by ascertaining all the cases within a population over a given period of time. The controls should then be randomly selected from the same population, differing in the outcome only. In the attempt to obtain perfect matching of cases, overmatching may be induced. This can reduce the validity or statistical efficiency of the case-control comparison, and increase the cost and the complexity of a study. Matching variables should be selected from those that have the strongest relationship to the study disease. As mentioned previously, alternatives to matching will need to be applied to the analysis. In fact, the use of a combination of matching and regression analysis generally removes more bias than matching alone.^{370,470,471} Post-stratification of variables which may contribute to bias within the study and confound the results, and the application of a regression model, may be used in the design, rather than stratified sampling or frequency matching. The former two methods offer well validated alternative approaches.^{370,470,471}

4.2.2.2 Performance bias. Performance bias can arise when the compared variables (or maneuvers) do not receive the same proficiency in the method of analysis (or administration). The attribute being studied may have been contaminated by the action of other known or unknown variables. One pertinent form of "performance" bias depends on the sensitivity and specificity of the methods of measuring the variables being examined. A negative result may be a false negative, as the method used may not be sensitive enough to detect a difference. This can arise from major variability of the characteristic over time, or the method used may not detect the appropriate abnormality. In order to minimise this particular bias, attempts can be made to keep the data collectors unaware of the main hypothesis. Also, the identity of the cases and controls may be hidden from those performing the measurement of the variables.^{370,470,471}

4.2.2.3 Detection bias. Detection bias can be a source of major bias in the case-control design. It arises when the outcome is detected unequally in the groups being

compared, but is mostly due to differences in medical surveillance. The surveillance of the groups to allocate cases and controls often requires diagnostic testing. Inequalities can occur in the performance of diagnostic tests and also in their interpretation. If the cases are ascertained following a particular diagnostic test or clinical event, and the diagnostic test is highly sensitive for the correct diagnosis, then ideally the same investigation should be undertaken in the control group. In practice this may be impractical, if not unethical, depending on the investigation concerned. The detection bias in the controls may be overcome by using another control group who have undergone the diagnostic test concerned, and who have a normal result.

The existence of a co-morbid outcome (another outcome that can influence the variable to be studied) may evoke increased medical attention, and influence the decision to undertake the diagnostic test used in the ascertainment of cases. The use of appropriately matched control groups, post-stratification of subgroups and/or regression analysis reduces this innate bias in the study design.^{370,470,471}

4.2.2.4 Transfer bias. Transfer bias may occur in the groups collected for the comparison of their results. After the outcome has occurred, there may be individuals who are lost to follow-up. Therefore, different types of outcome for the attribute being examined, may not be suitably represented in the data collected which is to be used for analysis. This particularly applies to case-control studies, where the outcome has occurred and the diagnosis of the outcome is the method of ascertaining the study group. Attrition of individuals with the outcome being examined, before inclusion in the case-control study, creates a group which is incompletely representative of the possible outcome group. This provides a source of bias difficult to counteract. Such individuals may possibly have differing results for the study variables than those found in the survivors. Their enforced exclusion may distort the results in the study group, compared with the original intact population.

4.2.3 Means of Reducing Methodological Bias

4.2.3.1 Sampling procedures. Unbiased ascertainment of eligible cases and controls is the prime aim of selection procedures within a case-control design. The procedure used for selection of a sample must ensure that each individual in the population has an equal chance of appearing in the study. Therefore, the subjects in this study:-

1. were not identified by the individual's exposure status in regard to the study factors, apart from the outcome (viz CHD).
2. are serial eligible cases arising within a defined period of time which were identified and asked to enter the study.
3. for the control group were selected and evaluated at the same time as the case group.
4. in the case and control group, individuals with conditions known to or which possibly may have predisposed to or against the study variable were excluded. Controls can be selected from a variety of sources to avoid the bias arising from differences in the composition of groups for comparison.

Nevertheless, some forms of bias cannot be controlled for in a case-control study.⁴⁷¹

As discussed above, a protopathic (first the pathology) bias refers to that arising when the outcome has already taken place but is not adequately recognised before a manoeuvre is allocated, or, the outcome has occurred and the consequence of this outcome is the attribute being studied.⁴⁷¹

4.2.3.2 Selection of controls. Matching of controls involves pairing one or more controls to each case on the basis of their similarity with respect to preselected variables. Such variables considered for matching are those that are risk factors for the study outcome, independent of the study attribute. Individual cases and controls can be pair-matched for factors, so that each are alike except for the factor under investigation. The controls were defined specifically to be socioeconomically equivalent to the case group. To avoid bias in comparing groups differing in composition, methods of selection such as adjustment procedures similar to age adjustment, were employed.

Some individuals in the control group may have subclinical CAD if the criteria for the clinical outcome is angiographic CAD. This study uses clinical criteria for the presence of CHD to select cases, and the absence of such criteria for the control group selection. Therefore the potential for misclassification of controls by disease status for angiographic CAD exists, but not for the presence of CHD. However, *such misclassification will dilute the differences between the groups that result in the development of CAD, but possibly may enhance the differences that result in the presentation in the form of CHD.* Some degree of this form of bias must occur in all epidemiological studies with clinical endpoints, and the awareness of this bias will add caution to the inferences derived from the observations.⁴⁷⁴

An alternative to matching is the specific comparison for each variable by stratifying for each factor level (stratified sampling). Post-stratification of variables and regression analysis are two main alternatives to matching used in the case-control study from this work.

4.2.4 Guidelines Used for the Case-Control Study Design

4.2.4.1 Establish research hypothesis. Platelet hyperreactivity is an aetiological factor in the pathogenesis of coronary artery atherosclerosis enhancing the premature development of the disease. This hyperreactivity results in a primary increase in platelet function and arises from an inherited platelet functional abnormality, and/or occurs secondarily from the effects on platelet function of the major risk factors for CAD.

Platelet hyperreactivity is an aetiological factor in the premature development of the acute clinical manifestations of the various forms of symptomatic CAD. Increased reactivity may be a primary abnormality, possibly inherited, and/or secondarily due to the effects of risk factors on platelet function.

4.2.4.2 Establish aims for the hypotheses. The aims of the research which arise from this hypothesis involve the investigation of platelet function in young individuals at increased risk for the development of CAD. It was necessary to determine if platelet dysfunction exists in young persons (age < 50 years) with proven CAD. If

measurable differences in platelet function were to be found, an evaluation of whether they were a secondary effect resulting from an association with other factors such as the major CHD risk factors was undertaken.

4.2.4.3 Define the study variables before commencement. Variables examined for an association with the disease outcome (platelet function variables) are defined in Chapters 2 and 3. It was particularly important to determine areas of bias due to possible confounding variables in order to define those variables required for matching, to establish admission criteria and define exclusion criteria.

4.2.4.4 Define admission and exclusion criteria. The locus in the time scale of the disease for this study was after the diagnosis, and *ipse facto*, after the clinical manifestation of the disease (unless the diagnosis was achieved before the clinical manifestation due to confounding variations of the medical surveillance of the disease). The baseline state of the disease in the study protocol was clinically manifest disease and the CAD was objectively evaluated by coronary angiography.

Admission criteria depend on the hypothesis and baseline state of the study disease, and include:-

1. Age less than or equal to 50 years of age
2. Documented myocardial infarction (pain with ECG changes and cardiac isoenzyme rise) or other clinical manifestation such as stable or previous unstable angina.
3. Angiographically proven CAD
4. Males only study groups
5. The cases were not selected if the diagnosis was made as an incidental finding, but only after presenting with clinical events.

The clinical status and the possible confounding factors of the patients were fully documented at the time of entry to the study. The exclusion criteria were predetermined and based on the same principles of exclusion as used in a randomised clinical trial (defined in Chapter 3).

4.2.5 Conclusions.

The evaluation of bias is an important requirement for planning and appraising the results of any case-control study. The studies reported in this Chapter will concentrate on some potentially major areas of bias in the design of the study. Other potentially confounding factors occurring in the analysis of the results which may contribute substantially to further bias in the outcome of the study are evaluated and discussed in Chapters 5 and 6, and Appendices 1,2 and 3.

4.3 PLATELET FUNCTION AND MYOCARDIAL ISCHAEMIA

4.3.1 Introduction

Pathological studies and clinical research have clearly demonstrated that platelets are

involved in the development of the acute obstructive ischaemic clinical syndromes of CAD.^{24,26,27,40,41,45,59,115,280,475} The question, however, of whether platelet activation and myocardial ischaemia are associated in those individuals with chronic stable CHD, or in those who have provokable myocardial ischaemia, is important for the selection of subjects when evaluating platelet function. It is possible that when blood flow increases in the presence of significant narrowing of the epicardial coronary arteries platelets are activated and aggregate more easily.⁴⁷⁶

Numerous methods of measuring platelet function have been used to investigate such a relationship. The release of platelet specific proteins in response to exercise induced myocardial ischaemia has yielded conflicting results.⁴⁷⁷⁻⁴⁸⁶ There is little available information on TXB2 levels in serum or plasma following exertional ischaemia, and those reports available give divergent results.⁴⁸⁷⁻⁴⁸⁹ An increased *in vitro* aggregation response to ADP following exercise has been a more consistent,⁴⁸⁹⁻⁴⁹¹ but not universal finding.⁴⁷⁹ These discrepancies require further clarification.

This section evaluates the effect of short term vigorous exercise, and exercise induced myocardial ischaemia, on specific measurements of platelet function in patients with CHD. Procedural standardisation, patient selection, medications, severity of CAD, and CHD risk factors are particularly emphasised. Variations in such factors may have contributed to previous conflicting findings. Moreover, this investigation aimed to determine whether, in the selection of subjects for the case-control study, the exclusion of those with exercise-induced myocardial ischaemia was necessary since platelet activation might be present in those with myocardial ischaemia but not in those without. This is also of clinical importance given the increased risk of sudden death during acute and unaccustomed exercise in individuals with CHD,⁴⁹²⁻⁴⁹⁴ and the demonstration of thrombosis and platelet aggregate formation in patients with ischaemic sudden death.^{26,27}

4.3.2 Methods

4.3.2.1 Patient population. The study consisted of 27 male subjects with proven CAD, who were asymptomatic, or had chronic stable angina. In 25 patients CAD was proven by coronary arteriography (at least one lesion with a luminal narrowing > 50%), and 2 patients had an AMI documented by new Q wave formation on their ECG and an associated rise in the creatine kinase MB isoenzyme. The mean age of the group was 53 years, ranging from 35 to 69 years. Stable angina was defined as exertional chest pain with no rest pain,⁴⁷² without an increase in severity or frequency, and with stable functional capacity. Group I consisted of 13 patients with exercise induced ECG changes of myocardial ischaemia. The 14 patients in group II had neither symptoms nor exercise induced ECG changes to indicate active exertional myocardial ischaemia. Specific exclusion criteria were applied to permit accurate interpretation of exercise ECG changes, to reduce confounding factors interfering with the platelet function studies, and to ensure maximum safety for the patients (Table 4.1).

4.3.2.2 Study Protocol. The study protocol was approved by the hospital ethics committee, and informed consent was obtained from each patient before participation. All drugs were ceased for at least 5 days before an exercise treadmill test, apart

from sublingual nitrates taken as required. Aspirin and nonsteroidal antiinflammatory drugs were ceased for at least 2 weeks. All patients underwent a maximum treadmill test, blood being taken immediately before and after the test. They then commenced routine antianginal drugs for 4 weeks, with no antiplatelet drugs of any form being taken. Of the original 27 patients, 24 again ceased all medications, apart from sublingual nitrates, for a minimum of 5 days before undergoing another treadmill test. The repeat exercise tests were to the same workload as the first, under the same conditions, and at the same time of day.

4.3.2.3 Exercise Protocol. The exercise protocol is described in section 2.12.

4.3.2.4 Laboratory Investigation. Blood was taken before and after each exercise test using minimal stasis and a 19 gauge "butterfly" needle with a 15 cm plastic tube. The measurement of plasma TXB₂, BTG, serum TXB₂, the PCR and the haematological indices are fully described in Chapter 2.

4.3.2.5 Coronary angiograms. The coronary angiograms were graded according to a semi-quantitative scoring method.²⁰⁷ A full description and validation of the methods is provided in Chapter 8.

4.3.2.6 Data Analysis. The differences between means of dependent samples were tested using the paired t-test, and a comparison between group means was made by the two sample t-test for all continuous variables apart from BTG and serum TXB₂, which had skewed distributions. Linear correlations were measured by Pearson's correlation coefficient. For plasma BTG and serum TXB₂, differences between paired samples were tested with the Wilcoxon matched pairs signed-ranks test, and correlations were measured using the Spearman rank correlation coefficient.

4.3.3 Results

4.3.3.1 Patient Profile (Table 4.2). Those with a positive exercise test were older and had a significantly lower left ventricular score (LVS) indicating fewer segmental wall motion abnormalities of the left ventricle. Those with a negative exercise test had a higher coronary stenosis score (CSS) due to a greater number having total coronary artery occlusions, but for the number of patients studied this difference did not attain statistical significance. There were no differences between the two groups in the amount of cigarette smoking, levels of the blood lipids, body weight and the extent of coronary atherosclerosis expressed as the coronary atheromatous score (CAS) and the mean CAS (mCAS). No patient had leg claudication, and all had palpable peripheral pulses.

4.3.3.2 Plasma BTG and TXB₂. There were no differences in the resting plasma TXB₂ levels between the two groups, nor was there a detectable change in plasma TXB₂ levels following maximum exercise. In 54% of the samples, the plasma TXB₂ levels were below the sensitivity of the RIA of 40 pg per ml. The measurement of small amounts of plasma TXB₂ (between 40 and 100 pg per ml) did not distinguish the ischaemic from the non-ischaemic group, nor the pre and post exercise samples.

The resting BTG was the same in both groups. Following exercise, there was a small but significant increase in the BTG level. This occurred in both groups in the second treadmill test, although only in the negative test group with the first exercise test (Fig 4.1).

4.3.3.3 Serum TXB2. There was a small but significant increase in the formation of TXB2 in serum following exercise. However, this increase only occurred in the ischaemic group, and was not observed in non-ischaemic group (Fig 4.2). The resting serum TXB2 levels in the ischaemic group was significantly lower than the level in non-ischaemic subjects (Fig 4.2).

4.3.3.4 Platelet count ratio (Table 4.3). The PCR was consistently lower in the non-ischaemic group at rest, but this difference was not statistically significant. A decrease in the PCR following the first treadmill test, was observed in the non-ischaemic group but not in the ischaemic group. This change was not reproduced in the subsequent treadmill test.

4.3.3.5 Blood Cell Indices (Table 4.4). There was a significant increase following exercise in the Hb, the Hc, the platelet count (PC) and the WCC, in both study groups. The values were the same for the ischaemic and non-ischaemic groups at rest and after exercise.

4.3.4 Discussion

For the appropriate conduct of a case-control study to evaluate platelet function in patients with stable CHD, it was necessary to determine if symptomatic or inducible ischaemia influenced platelet function since this could be a potential source of bias. A clinically important role has been clearly demonstrated for the involvement of platelets and thrombus in the acute occlusive ischaemic syndromes of unstable angina, AMI and sudden ischaemic death.^{24,26,27} However, numerous studies of the role of platelets in effort induced myocardial ischaemia have produced conflicting results. Not only does a cause-effect relationship between platelet activation and exercise induced ischaemia remain unresolved, but indeed, even the presence of measurable changes has not been consistently demonstrated.

In addition to the requirements of conducting the case-control study, this study allows an evaluation of *in vivo* platelet function with exercise in patients with exertional ischaemia and therefore may contribute to an understanding of mechanisms resulting in the increased risk of sudden death associated with exercise in individuals with CHD. Indeed, the results of this study indicate that during exercise-induced myocardial ischaemia in a stable CHD population, platelets are not activated as assessed by the parameters measured. In particular, measures of *in vivo* platelet function were unaffected by the presence or absence of exertional myocardial ischaemia. The results support previous work showing no relationship between exertional ischaemia and plasma TXB2⁴⁸⁸ or plasma BTG levels.⁴⁸¹

An increase, however, in plasma BTG following exercise has been previously documented.⁴⁹⁵ In the present study, the plasma BTG clearly increased in those individuals undergoing maximal exercise and the increase in the ischaemic group

was attenuated. Those with negative exercise tests were able to exercise to workloads which were maximal or near maximal for their age. In contrast, those with evidence of myocardial ischaemia attained lower levels of exercise, were limited by their cardiac response to exercise, and were below their predicted achievable workload. If the increase in BTG is a non-specific response to exercise itself, those with limitations due to ischaemia may not perform sufficient work to elicit an increase in BTG, even though the workload was sufficient to cause an increase in the platelet count, Hb and Hc.

The effect of exercise on the PCR in patients with CHD remains unresolved. A significant overall decrease in the PCR following exertion in the first treadmill test could not be reproduced in the third test under similar conditions. The reason for this is not apparent, but may be due to a training effect occurring between the two treadmill tests, or alternatively, to variability in platelet reactivity over time. The resting PCR in patients with CAD was previously shown to be significantly lower than in normal subjects.⁴⁹⁶ In that same study, following short-term strenuous exercise, the PCR decreased in the CAD patients irrespective of the presence of ECG changes of myocardial ischaemia.⁴⁹⁶ In our study, the decrease in the PCR in the first treadmill test predominantly occurred in the non-ischaemic group. The PCR did not decline in those with exertional ischaemia, indicating that platelets do not become more reactive following exertional ischaemia, as measured by this method. It is possible, however, that platelet aggregation measured by whole blood aggregation methods may be influenced by pacing induced tachycardia,⁴⁷⁶ although this is controversial.^{44,497-501}

Measurement of TXB2 is used as an estimate of the activity of platelet prostaglandin metabolism. Serum TXB2 provides a measure of the platelet response to endogenous agonists during *ex vivo* clotting. These agonists may be present before the clot is formed and/or produced during its formation. In this study, those individuals who had objective evidence of ischaemia, produced less TXB2 in serum, but had a modest increase following exercise. This increase was not apparent in the non-ischaemic group, who also had significantly higher resting levels of serum TXB2. Reuben and colleagues did not find an increase in serum TXB2 after exercise in a group of patients with CAD who had a positive exercise test for myocardial ischaemia.⁴⁸⁹ However, their exercise test was a symptom limited treadmill test, exercise being ceased with the first onset of angina. All patients were receiving conventional anti-anginal medications which were continued throughout the study, and it is possible that these drugs prevented any increase in serum TXB2.⁵⁰² The plasma TXB2 levels did not relate to exercise or exercise-induced ischaemia. The plasma TXB2 levels however may not reflect true circulating TXB2 levels, its presence being due to technical factors, such as release during blood collection.⁵⁰³

An explanation of the changes observed in the serum TXB2 levels cannot be determined from this study. One possibility, however, is that increased substrates are formed during exercise induced ischaemia which will increase prostaglandin metabolism by those cells involved in the clotting process. A similar increase in prostaglandin activity may occur during reversible platelet aggregation and/or non-obstructive thrombus formation on ruptured atheromatous plaque, and so increase localised thrombus formation.

A lower serum TXB2 was evident in the ischaemic group. This reduced production of TXB2 with clotting in patients with known inducible myocardial ischaemia has also been observed by others.⁴⁸⁹⁻⁵⁰⁴ It is possible that intermittent platelet activation may be occurring in patients with active myocardial ischaemia, depleting the potential for serum TXB2 production. Nevertheless, there is no evidence to support this suggestion from those measures of *in vivo* platelet activation in this population with exertional ischaemia. There was no significant difference between platelet counts or cholesterol levels in the two groups to account for the difference.^{504,505} The differences found in platelet function cannot be attributed to the severity of CAD. There was no significant difference in the extent of coronary atheroma in the two study groups as measured by the mean CAS. Even though there was a tendency in the group with a negative exercise test to have a higher stenosis severity score (mean CSS), *in vivo* platelet reactivity was the same as for the ischaemic group. The group with positive exercise tests, who were older, may have more extensive peripheral vascular disease to account for the differences in serum TXB2. However, there were no clinically detectable differences, and again, measures of *in vivo* platelet activation were the same in both groups. The higher serum TXB2 in the non-ischaemic group may be age related, given the significantly younger age of the non-ischaemic group.

The haemoconcentration, increase in white cell numbers and rise in peripheral platelet numbers which occurred with exercise, have been previously well documented and substantiated.^{347,506} The changes in the blood cell indices occurred in both groups, with greater changes occurring in those attaining higher workloads.

4.3.5 Summary

1. In light of the foregoing observations, in order to detect a measurable difference in *in vivo* platelet function between young males with CHD and a normal control group, the exclusion of subjects with ongoing measurable myocardial ischaemia is not required.

2. Short-term maximally strenuous exertion may cause activation of platelets irrespective of the presence of ischaemia. The avoidance of such exertion in routine unmonitored activity by CHD patients is advised for many important cardiological reasons, and possible platelet activation may be an additional factor. Whether antiplatelet drugs will help to reduce the incidence of adverse consequences associated with such exercise remains to be determined.

3. Exercise-induced myocardial ischaemia does not appear to activate circulating platelets, nor are previously activated circulating platelets associated with exertional ischaemia.

4. It is possible, however, that including subjects with exertional ischaemia will reduce the possibility of detecting a difference in serum TXB2, since those with exertional ischaemia may produce less TXB2. Therefore, if a difference is demonstrated between the case and control group to be evaluated, then it is more likely to be a true difference.

4.4 PLATELET FUNCTION AND ANTI-ISCHAEMIC MEDICATIONS

4.4.1 Introduction

An inhibitory effect of beta-blockers on platelet function has been clearly demonstrated *in vitro* at relatively high concentrations⁵⁰⁷⁻⁵¹⁰ and also at therapeutic concentrations.⁵¹⁰ However, conflicting data exists as to the effect of orally administered therapeutic doses of beta-blockers in normal individuals,⁵⁰⁹⁻⁵¹⁵ and in those with CHD.^{483,491,516} *In vitro* inhibitory effects of calcium entry blockers on platelets have also been well documented,⁵¹⁷⁻⁵²⁶ although controversy has also arisen regarding evidence for an effect in persons receiving oral calcium entry blockers.^{521,527-529} *In vitro* studies have demonstrated different modes of action for these two group of drugs on platelets. Thus, a combination of beta-blockers and calcium entry blockers may be sufficiently effective in suppressing platelet activity *in vivo* to be measurable. Furthermore, *in vitro* findings may not reflect the *in vivo* effects because of artifacts inherent in the preparation of PRP⁵³⁰ and metabolic changes in other blood components which may influence platelet activation such as the release of ADP from red blood cells.³⁴⁹ Clearly, *in vitro* findings cannot be equated unquestionably to the *in vivo* situations, which must be evaluated specifically and independently. The inhibition of *in vitro* platelet function by nitrates has also been the subject of frequent reports.⁵³¹

In the selection of subjects for a case-control study, it is of importance to determine whether or not it is necessary to stop cardiac medications used by the volunteers. This study therefore examines the *in vivo* effects of a combination of a beta-blocker (metoprolol) and calcium entry blocker (nifedipine) on platelet function at rest and after exercise. The effect of these anti-ischaemic drugs on platelet function before and after exertional ischaemia was also evaluated.

4.4.2 Methods

4.4.2.1 Patient population. The study population is the same group described in section 4.2.2.1, and the results constitute part of a single study.

4.4.2.2 Study Protocol. The methods have previously been described in full detail²⁹⁸ and in section 4.2.2.2. Importantly, all drugs were ceased for at least 5 days before the first exercise test. Aspirin and nonsteroidal antiinflammatory drugs were ceased for at least 2 weeks. Blood was sampled immediately before and after each maximal exercise test. After the first exercise test, 26 of the volunteers commenced metoprolol 50 mg twice daily and nifedipine 10mg three times daily for approximately 4 weeks. The patients were then exercised at the same time in the morning, 1.5 to 2 hours after taking the medications. Then 24 patients stopped the medications for a minimum of 5 days before undergoing a third treadmill test. Three patients withdrew for reasons unrelated to the study or drug side effects. All subjects performed a maximum treadmill exercise test.

4.4.2.3 Investigations. The laboratory investigations and angiographic scoring details are described in Chapters 3 and 8 respectively.

4.4.2.4 Data Analysis. The data analysis methods are described in Chapter 2 and section 4.2.2.

4.4.3 Results

4.4.3.1 Plasma BTG and TXB2. The resting BTG level was slightly higher in the group with positive exercise tests whilst not on medication, but the difference was not significant (Fig. 4.4). Following exercise there was a small but significant increase in the BTG levels, which occurred in both the positive and negative stress test groups in the second and third treadmill test, although only in the negative test group for the first exercise test (Fig. 4.4). A significant increase in plasma BTG level in both groups following exercise was not altered by the study medications (Fig.4.4).

No difference was observed in resting plasma TXB2 levels between the two groups, nor were there detectable changes in plasma TXB2 levels following maximum exercise. The medications had no measurable effect on the plasma TXB2 levels before or after exercise.

4.4.3.2 Serum TXB2. A modest but significant increase in TXB2 levels with exercise was observed in both tests without medications, and this increase was diminished in the presence of the study medications (Fig. 4.5). This small but significant increase in the formation of TXB2 in serum following exercise occurred in the ischaemic group, but not in non-ischaemic group (Fig. 4.5). There was also a significantly lower level of the resting serum TXB2 in the ischaemic group compared to the non-ischaemic subjects (Fig. 4.5).

4.4.3.3 Platelet count ratio. The PCR decreased following exercise in the initial exercise test without medications. This decrease was not reproducible in the third exercise test without the study medications. No significant change was demonstrated in the PCR whilst subjects were on medications (Fig. 4.3). The PCR was consistently lower in the non-ischaemic group, but this difference did not attain statistical significance. The mean PCR at rest was the same in subjects with or without medications, and when they were divided into positive or negative exercise groups (Fig. 4.3).

4.4.3.4 Blood Cell Indices. The medications had no affect on these variables at rest or after exercise (Table 4.4).

4.4.4 Discussion

Anti-platelet therapy has been clearly identified as having a protective effect following acute ischaemic coronary events.^{97,98,104} An *in vitro* inhibitory effect on platelet function by both beta-blockers and calcium entry blockers has been well established.^{507-509,510,517,519-526} The *in vivo* effects on platelets of these classes of drug are less well defined and controversial.^{509,511-515,521,527-529}

In the previous section, evidence was provided that in patients with stable CHD exertional myocardial ischaemia does not activate circulating platelets.²⁹⁸ This result

confirmed previous reports⁴⁸⁵ and has subsequently been verified by another study.⁵³² Predictably, specific anti-platelet drugs have no significant effect on objective measures of exertional myocardial ischaemia in patients with chronic stable angina.⁵³³ However, our observations suggested that brief strenuous exercise with the induction of myocardial ischaemia creates a milieu in whole blood which leads to increased TXB2 release during *in vitro* thrombus formation.²⁹⁸ Unstable angina is associated with disease progression,^{114,534} intracoronary thrombus formation,^{23,26,115,535,536} and also the production of increased amounts of excreted thromboxane metabolites.^{537,538} Moreover, pathological evidence exists for the involvement of thrombus in disease progression.^{34,539}

Given that thrombus formation may be involved in the formation of new stenoses and occlusions, a mechanism for drugs to prevent such lesion development is to contribute to the inhibition of thrombus formation. The combination of a beta-blocker and a calcium entry blocker is more effective in the prophylactic treatment of symptomatic CHD than the use of single drugs.⁵⁴⁰ Beta-blockers reduce the incidence of recurrent CHD events after an AMI, including the thrombotic event of recurrent MI.^{541,542} However, calcium entry blockers have not as yet been shown to be effective against further obstructive ischaemic events after an acute myocardial infarct,⁵⁴³ except possibly in specific subsets of patients.⁵⁴⁴ Yet, as discussed, they appear to lessen the development of new occlusive lesions in patients with CHD.^{545,546} Some understanding of the mechanisms of the anti-ischaemic effect of these drugs has been provided following extensive investigation,⁵⁴⁷ although the mechanism(s) for the possible secondary preventative effect has not been clarified.^{546,548} An inhibitory action on platelet reactivity and a reduction in a continuing thrombotic tendency, is one possible contributing mechanism that requires further investigation.

The results fail to demonstrate any effect on specific measures of *in vivo* platelet function by the combination of a beta-blocker and calcium entry blocker. Plasma TXB2 and BTG levels at rest did not change. A modest increase in plasma BTG levels with exercise was unaffected by the addition of the medications or the presence of exertional ischaemia. An initial significant difference between the PCR before and after exercise was not reproducible in the repeat test without treatment. It is possible that the repeat test without medication by chance failed to demonstrate a true difference. However, this is probably unlikely, the repeated measures of the PCR may represent the phenomenon of regression to the mean.⁵⁴⁹ In the absence of a control or placebo group, the average of two or more measures for classification of the initial variable for comparison can be used.⁵⁵⁰ Using this method to reduce the effect of regression in the PCR measures, that is averaging the values for the first and third measurement when not on treatment, then there is no difference before and after exercise, and with and without treatment.

Following exercise, the serum TXB2 levels increased significantly in the group with a positive exercise test but not in the group with a negative exercise test.²⁹⁸ Although the increase in serum TXB2 level was small, the change within the group was clearly attenuated by the drug combination. Serum TXB2 measures prostaglandin activity of blood cells during thrombus formation *ex vivo*. The production of prostaglandin metabolites will be influenced by platelet agonists present in the blood, or formed

during thrombus production. Suppression of TXB2 formation may arise from a number of factors, including a reduction in agonist concentration, reduced platelet prostaglandin metabolism, and/or reduced platelet response to endogenous agonists. The beta-blocker propranolol *in vitro* appears to have inhibitory activity on BTG release and TXB2 production by whole blood and on spontaneous clotting at concentrations near the therapeutic range.⁵¹⁰ This may be due to an inhibition of collagen and thrombin-induced platelet aggregation and TXB2 synthesis.⁵¹⁰ The present findings are consistent with these *in vitro* observations, although the mechanism of the effect of the drug combination on platelet prostaglandin activity measured *ex vivo* cannot be deduced from this study. Inhibition of platelet function *in vivo* following the infusion of isosorbide mononitrates at increasing doses has been reported and includes a reduction in TXB2 production in PRP following agonist induced aggregation.⁵³¹ The degree of inhibition was related to the rate of the infusion and it was concluded that the variability of the functional platelet response was related to the haemodynamic response.⁵³¹ Eight of 11 patients in the study population had stable CHD with exercise ECG changes of myocardial ischaemia but characterisation according to the presence of inducible ischaemia was not reported. However, the observations suggest a relationship to the decrease in BP. Therefore it is possible that the abolition in the increase in serum TXB2 is related to the decrease in BP response to exercise whilst on the study medications rather than a direct effect on the platelet.

The explanations for the increased serum TXB2 production after exercise in ischaemic patients cannot be determined from the current study. However, it would appear from the lack of response seen with the measures of *in vivo* platelet activity, that this increase is unlikely to be due to an increase in circulating platelet activation. It is when platelets are activated during thrombus formation *ex vivo*, following exertional myocardial ischaemia, that a capacity for increasing thromboxane production occurs. Should this increase in prostaglandin production occur during thrombus formation *in vivo*, then a greater stimulus would be available for propagation of thrombus. Whether or not the combination of drugs contributes to a reduction in platelet prostaglandin activity *in vivo* and diminished thrombus propagation during endothelial desquamation, or atherosclerotic plaque rupture, can only be speculative.

The results of the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT) study confirmed previous experimental studies demonstrating a significant reduction in newly formed coronary artery lesions (stenoses and occlusions) in patients on nifedipine compared to placebo, especially in the presence of early CAD.⁵⁴⁶ A proposed explanation, which will be briefly stated and elaborated on subsequently, ensues from the proposal that disruption of small atherosclerotic plaques with subsequent mural thrombosis and fibrotic organisation of thrombus seems to contribute to the progression of atherosclerosis.³² Firstly, nifedipine reduces the development of new lesions.⁵⁴⁶ Our results show that the combination of nifedipine and metoprolol reduces the amount of thromboxane produced during whole blood clotting *ex vivo*. Progression of CAD involves new lesion formation that is not entirely related to the underlying atherosclerosis, implicating additional mechanisms such as thrombosis. Pathological evidence exists for the involvement of thrombus in disease progression.^{34,539} and clinical syndromes (unstable angina and AMI) are associated with new lesion development, in association with a fissured

plaque, and increased thromboxane production. Moreover, increased TXB₂ production occurs in whole blood clotting in the presence of provoked ischaemia.²⁹⁸ Therefore, a reduction in thromboxane production might reduce the likelihood of the production of those new CAD lesions that involve thrombosis.

Such a scenario implies that disease progression, particularly the formation of discrete lesions, has a major non-atherosclerotic component, namely thrombus formation. The following brief review of disease progression, as interpreted from angiographic studies, supports this concept. Despite being able to say which areas of the vessel have a common site of progression,⁵⁵¹ the use of previous angiograms to predict which vessels in an individual would be involved in progression has not been reliable.⁵⁵² In fact, not only does knowledge of the initial pathology not provide the ability to accurately predict progression, but the frequency of progression does not appear to be related to the initial number of vessels with greater than 50% luminal obstructions or luminal occlusions.⁵⁵² Furthermore, although angiographically normal coronary arteries have a low rate of progression,⁵⁵³ those with some intraluminal disease without a significant luminal obstruction have a rate of progression almost equivalent to those with established CAD.⁵⁵⁴ Undoubtedly progression occurs in old existing atheromatous lesions. However, new areas of significant narrowing frequently occur in previously apparently normal segments⁵⁵⁵ and the progressive nature of the disease is quite episodic.⁵⁵⁶ Indeed, progression of occlusive CAD occurs commonly in areas that have previously been minimally diseased in patients without initially clinical disease requiring a PTCA or coronary bypass grafting.^{555,557,558} Anatomical studies have shown that a substantial percentage of lesions associated with coronary artery thrombosis and AMI are moderate in severity.^{12,559}

In summary, a high incidence of progression of CAD in vessels that are normally minimally diseased has been well documented.^{20,555,557,558,560} although the progression to total occlusion also occurs frequently in very severely narrowed lesions.^{21,43,555} The progression of coronary atherosclerosis is a highly unpredictable process that follows a non-linear course and information about its dynamics derived from sequential coronary arteriograms hardly improves prediction of future progression.⁵⁶¹ Knowledge of the initial pathology does not allow accurate prediction of where new lesions will occur.^{551,562,563} In addition, there appear to be time dependent and time independent factors.^{551,564} The time dependence does not appear to be present for those developing unstable angina and progression compared with individuals having chronic stable angina and disease progression.

4.4.5 Summary

1. There is no evidence of activated circulating platelets following exertional ischaemia and no measurable effect of the drug combination of metoprolol and nifedipine on *in vivo* measures of platelet activity.

2. An increase in prostaglandin biosynthesis *ex vivo* in whole blood associated with exercise induced myocardial ischaemia is attenuated by a combination of a beta-blocker and a calcium entry blocker. The drugs may produce their effect by reducing myocardial ischaemia, BP or, alternatively, by directly decreasing blood

cell prostaglandin formation.

3. In addition, it may be possible that a reduction of whole blood prostaglandin activity during thrombus formation in individuals with myocardial ischaemia may contribute to the protective effect of these drugs against acute, and transient obstructive thrombotic coronary episodes.

4. For evaluation of platelet function in a cross-sectional study, such a potentially confounding factor will not be a major bias. For patient safety, all cardiovascular medications were not ceased beforehand. This allowed the serial selection of patients with stable premature CHD, rather than waiting and only selecting patients without medications, or who are sufficiently stable to ethically stop medications.

4.5 VARIABILITY OF SERUM TXB2 LEVELS AND INTERACTION WITH OTHER PLATELET FUNCTION TESTS AND BLOOD CELL INDICES

4.5.1 Introduction

The significance of variability in the production of thromboxane in clotting whole blood, and indeed, whether there is any variability at all, remains controversial. In some clinical syndromes with altered platelet activation, the capacity of platelets to form thromboxane in serum remains unchanged, although biosynthesis *in vivo* appears to be increased.^{332,565,563} This is not surprising given that the normal rate of thromboxane synthesis *in vivo* only represents a fraction (probably less than 0.1%) of the capacity of platelets to generate thromboxane.⁵⁶⁵ Furthermore, pharmacological inhibition of serum thromboxane formation must be virtually complete before thromboxane dependent platelet activation is influenced *in vivo*.⁵⁶⁵ The use of serum TXB2 measurements may be of value as a guide to the adequacy of dosage with platelet-inhibitory drugs by providing a measure of cyclo-oxygenase inhibition.^{503,565} Indeed, the inhibition of whole blood TXB2 production by platelet-inhibiting drugs may give a measure of platelet function inhibition in individuals with increased platelet activation and aggregability when exposed to an appropriate stimulus. Other than these latter two possibilities, no clinically useful role for the measurement of serum TXB2 has been elucidated.

4.5.1.1 **Whole blood clotting and thromboxane B2 formation.** Variation in the maximum amount of TXB2 produced by platelets during clotting *ex vivo* may have no clinical relevance. Nevertheless, *in vitro* experiments indicate that the maximum amount of TXB2 that can be produced may vary. For example, exogenous thrombin will further increase the amount of TXB2 produced.⁵⁶⁶ Moreover, when human platelets are treated with thrombin to induce the release of their alpha and dense granule constituents, additional TXB2 can be formed in response to other platelet agonists, albeit less than by platelets not depleted of their granule contents.⁵⁶⁷ In contrast, arachidonic acid induced TXA2 formation does not differ between thrombin-treated platelets and non-treated platelets,⁵⁶⁷ and the addition of extra thrombin produces significantly less TXB2 by thrombin-treated platelets than by control

samples.⁵⁶⁸ Furthermore, collagen stimulation leads to reduced TXB2 formation in degranulated platelets, but more TXB2 is produced than when thrombin is used as the agonist with the same platelets.⁵⁶⁷ Pretreatment of platelets with thrombin does not lead to recovery with respect to thrombin inducible TXB2 synthesis when platelets are incubated in the presence or absence of exogenous arachidonic acid.⁵⁶⁸ Indeed, impaired thromboxane synthesis in pre-activated platelets may result from an agonist-specific, irreversible desensitisation to thrombin.⁵⁶⁸ These experiments indicate that the production of thromboxane is, in part, dependent on prior exposure to platelet agonists, in particular thrombin.

Therefore it would appear that substrate availability and/or degree of agonist stimulation, and not the enzyme system, is the limiting factor in the production of TXB2 in serum. The observations demonstrate the very real potential for platelet agonists and metabolic substrates of thromboxane to influence the ability of platelets to form TXB2 leading to variability in TXB2 formation in association with clotting.

4.5.1.2 Thromboxane Synthesis in Different Diseases. Diabetics may have an altered capacity to generate TXB2 in clotting whole blood.⁵⁶⁹ Subjects with non-insulin dependent (NIDDM) and insulin dependent diabetes mellitus (IDDM) have both been found to have a reduced capacity to synthesise TXB2 in response to endogenous stimuli released during whole blood clotting with the synthesis of TXB2 being influenced by the degree of diabetic control.⁵⁶⁹ Others however, have found that platelets obtained from subjects with NIDDM synthesise significantly higher amounts of TXB2 than those with IDDM and matched controls.⁵⁷⁰ Yet again, in another study, the ability to generate TXB2 did not differ between diabetics and controls.⁵⁷¹ The platelets from diabetics with microangiopathy or from diabetics taking oral hypoglycaemic agents produced reduced amounts of TXB2 during clotting.⁵⁷¹ Additionally, a wide variability is present in all the groups studied, and there is a significant overlap in values for individuals.

Reduced platelet TXB2 formation has been noted in patients with renal failure.^{566,572} In uraemic patients, there may be up to a 60% decrease in TXB2 formation in clotting whole blood.⁵⁶⁶ Exogenous thrombin failed to restore normal TXB2 production in uraemic patients, whereas TXB2 production increased upon the addition of thrombin to serum in normal subjects. These characteristics are consistent with a functional defect of the enzyme cyclooxygenase. In another study, patients with chronic renal failure produced less serum TXB2 than age- and sex-matched controls.⁵⁷²

An important influence on the production of TXB2 in serum appears to be the other cell constituents in blood. For example, the reduction in TXB2 synthesis observed in association with renal impairment occurred in the presence of a lower Hc and a different platelet count.⁵⁷² This study indicates that the decrease in TXB2 production may not be due to a cyclooxygenase defect, but rather to differences in other blood parameters which could influence TXB2 production, including Hc and platelet count.⁵⁷² Indeed, there is evidence to suggest that TXB2 formed during clotting should be divided by the platelet count.^{572,573} It would appear that there is a dependence of TXB2 production in PRP on the platelet count.⁵⁷³ The TXB2 production in clotting whole blood in normal subjects also appears to be related to the Hc and the

platelet count.⁵⁷²

Soon after an AMI, male patients with no other diseases present, when compared to an age- and sex- matched control group, show a large decrease in platelet cyclooxygenase end-products formed during clotting of whole blood.⁵⁷⁴

4.5.1.3 Intra-individual variability in normals. Some studies suggest that in normal subjects there is no clinical or statistically significant correlation between serum TXB2 levels and age, BMI, BP, tobacco use, cholesterol level, triglyceride level, or blood glucose level.^{504,575} However, as indicated above, it would appear that the serum TXB2 level is related to the number of platelets in whole blood.^{504,572,575} Furthermore, repeat blood sampling over successive days in healthy subjects demonstrates an intra-subject variability in serum TXB2 levels of 14+/-5%.⁵⁷⁶

4.5.1.4 Influence of extrinsic factors on serum TXB2. The formation of TXA2 in whole blood, measured as its stable metabolite TXB2, reflects the capacity of platelets to form TXA2 in response to thrombin and other endogenous platelet agonists. TXA2 is the main product of cyclooxygenase metabolism in platelets,^{577,578} but TXA2 is also produced by polymorphonuclear leukocytes⁵⁷⁹ and monocytes.⁵⁸⁰ However, thrombin formed during blood coagulation preferentially stimulates platelets compared to monocytes,⁵⁸¹ and the amount of TXB2 produced directly correlates with the whole blood platelet count in normal individuals.^{504,572}

Serum TXB2 levels have been used as an *ex vivo* measure of platelet activation in a number of medical conditions.^{566,569} However, the capacity of platelets to form TXB2 *ex vivo* (measured as the levels of TXB2 in clotted whole blood) may not differ in various clinical conditions associated with platelet activation *ex vivo*.^{563,582} If the maximum amount of TXB2 formed in clotted whole blood is fixed and independent of the biological milieu and clinical conditions, it would be an inappropriate measure for the investigation of differences in platelet function between different clinical groups.

4.5.1.5 Summary. Whether the differences in serum TXB2 levels between the control and case groups (discussed in Chapter 6) have any relationship to differences in platelet function/morphology, or whether they are due to factors in the blood which influence platelets is uncertain. For example, it is possible that substrate availability and/or an agonist effect may cause the variability.⁵⁶⁶⁻⁵⁶⁸ There is ample evidence that serum TXB2 production may be influenced by the platelet count and Hc,^{572,573} by renal impairment,^{566,572} by diabetes mellitus⁵⁶⁹⁻⁵⁷¹ and following recent AMI.⁵⁷⁴

Because of these observations, we have reexamined the possible contribution to the production of TXB2 by other cells involved in clot formation as a possible cause of the differences found between the case and control groups. Only individuals without diabetes, renal impairment and a recent AMI can be included in the study populations evaluated, reducing the confounding effects on serum TXB2 production and allowing a more valid evaluation of the influence of blood cell indices on serum TXB2 production. In addition, if the maximum amount of TXB2 remains fixed and

not variable for an individual, then a relationship between the amount of TXB2 formed and measures of platelet reactivity would not be expected. Therefore, the relationship between the synthesis of TXB2 in clotted whole blood and standard measures of platelet activation in the study groups was also examined.

4.5.2 Methods

4.5.2.1 Patient population. The three groups evaluated in this analysis were the young male group of patients with CHD (the case group described in Chapter 3), the young male control group without clinical CHD (the control group described in Chapter 3) and an older group with stable CHD involved in the exercise study (described in sections 4.2.2.1 and 4.2.3.1). The exercise group has been included in this analysis because of the potential influence of cardiovascular drugs on serum TXB2 production.

4.4.2.2 Laboratory and clinical methodology. Methods used in this evaluation are described in Chapter 2.

4.5.2.3 Statistical analysis. An analysis of covariance was used to demonstrate the effect of specific uncontrolled variables, which are intrinsic to the subjects, on the mean values of variables used in the comparison of groups. As with the regression models used, the analysis of covariance provides a statistical rather than an experimental method to control and adjust for the effects of one or more uncontrolled variables.⁵⁸³ The influence of the covariate is removed using a linear regression method, residual sums of squares are then used to provide variance estimates that are then employed in tests of significance.⁵⁸³ Adjusted means of the dependent variable are obtained, and the adjusted mean shows which part of the variation in the means between groups remains when the variation due to the covariate is removed.⁵⁸³ Necessary in the analysis is the calculation of the within group variable variance and the between group variable variance.

In this analysis, the covariates used were obtained simultaneously as the dependent variable, using the same blood sample. In addition, the variables assessed are intrinsic attributes of the subjects studied. Furthermore, a number of assumptions are made in this analysis. In view of the lack of overt familial forms of hyperlipoproteinaemia or other known genetic influences on the dependent variable, it will be assumed that the differences between the groups are not genetically determined for the factors analysed. A direct causal effect is assumed between the dependent variable and the covariates. The statistical assumption for the presence of homogeneous regression coefficients is also made in this method (MANOVA in the SPSSX).

4.5.3 Results

4.5.3.1 Platelet count. There was no measurable relationship between the platelet count and plasma TXB2 levels, plasma BTG levels, Hb, Hc or WCC in the young normal (control) group. A very small but consistent positive relation between the platelet count and the serum TXB2 level was present in the groups with CHD, but not in the young normal group (Table 4.5).

4.5.3.2 Serum Thromboxane B2. The amount of TXB2 formed after incubation for 1 hour at 37°C had a significant negative relationship with the PCR in the older CHD group and in the young control group (Table 4.5). Although the correlation with the PCR is only small, there is clearly a reproducible association in these groups but not in the young CHD group. Besides the platelet count, the serum TXB2 level correlated with Hb, Hc and WCC in the two CHD groups but not in the control group (Table 4.5). The serum TXB2 level correlated negatively with the BTG level in the normal group ($r=-0.38$, $p<0.005$), although the serum TXB2 level was not related to the plasma TXB2 or plasma BTG levels in patients with CHD.

Using a stepwise regression model, with serum TXB2 as the dependent variable and age, Hb, Hc, WCC and PC as the independent variables, only the PC and WCC remained significantly and independently associated with serum TXB2 levels in the young case group, while the WCC was the only variable associated with serum TXB2 level in the older CHD group (Table 4.6). There were no significant associations in the control group for the regression analysis using the same variables.

An analysis of covariance was performed using MANOVA (SPSSX), with the dependent variable being the serum TXB2 level, the covariates being Hb, Hc, PC and WCC, and the case and control groups being analysed as factors, that is, as categorical variables defined in two groups. In fact, these two groups were analysed together by the analysis of covariance method. The cases and controls were considered as categorical values and defined as two factors in the analysis of covariance. The adjusted means for serum TXB2 values, that is the means adjusted for the effects of the covariates Hb, Hc, WCC and PC, remained very different for the case group (adjusted logarithmic mean being 4.56 ng/ml) compared to the control group (adjusted logarithmic mean being 1.33 ng/ml). The summary of the analysis is presented in Table 4.7. The means are expressed as the logarithm since the serum TXB2 value was originally logarithmically transformed to obtain a normal distribution to allow Gaussian statistical methods to be used.

4.5.3.3 Platelet Count Ratio. The PCR is a measure of *ex vivo* platelet reactivity. A consistent and significant relationship between the PCR and other blood cell indices was only observed in the older CHD group (Table 4.8), but not in the young CHD group, except for a negative relationship with the WCC ($r=-0.45$, $p<0.001$). The PCR correlated negatively with serum TXB2 levels in the control group and in the older CHD group. In order to evaluate the importance of other cells in determining the association between the serum TXB2 level and the PCR in the older CHD group, the correlation was further examined using partial correlations. This method adjusts for the effects of independent variables on any of the measured zero order partial correlations. The association between the serum TXB2 level and the PCR diminished after adjusting for Hb, Hc, WCC and PC, although only to a small degree (Table 4.9).

4.5.3.4 In vitro platelet aggregation. A positive association was demonstrated between the reactivity of platelets in PRP to specific platelet agonists and serum TXB2 values in both the control group and the case group. In particular, in the control group, a strong consistent association existed between platelet aggregation and the amount of TXB2 formed (Table 4.10). This measurement was not performed

in the older group with CHD.

4.5.3.5 Influence of cardiac medications on the associations. In the older CHD group, the associations observed between the serum TXB2 and the blood cells, hB and Hc were not affected by the use of nifedipine and metoprolol (Tables 4.5, 4.8 and 4.9).

4.5.4 Discussion

The question of whether serum TXB2 levels reflect an intrinsic variation in platelet reactivity remains controversial, since such variation may simply be due to differences in the number of blood cells contributing to its formation. For this reason we examined the relationship between serum TXB2 levels, blood cell indices and measures of platelet reactivity. In this respect, the type of patient with CHD which is evaluated is of prime concern since there appears to be a difference in *in vivo* platelet reactivity if one of the clinical complications of coronary atherosclerosis is present.

4.5.4.1 TXB2 production and the clinical circumstances. Patients with stable CHD appear not to have persistent activation of circulating platelets^{45,298} in contrast to those with unstable angina or an AMI.^{45,46} Therefore the latter groups would be more appropriate to evaluate the relationship between serum TXB2 levels and *in vivo* platelet activation and determine if variability in serum TXB2 levels quantitatively reflects platelet reactivity. In practice however, the use of intravenous drugs, aspirin, invasive investigations and indwelling intravenous cannulae introduce factors which themselves significantly alter platelet function making it ethically difficult to study this group because of the need to withdraw such factors. Therefore, we have utilised the concept of reproducibility of results under the same conditions to validate positive associations, particularly when multiple statistical comparisons are being made. Also of particular importance, the study was designed to limit exogenous factors which have the potential to affect platelet function.

4.5.4.2 TXB2 production and cardiac medications. Prior to discussing the potential variability of serum TXB2 levels, it is necessary to address the issue of a possible major confounding factor in measurement of platelet function, namely the effect of cardiovascular drugs. This problem has been discussed in section 4.2. One group (an older CHD group with stable clinical disease) was examined in whom all medications were stopped to see if any bias may arise from the effect of cardiovascular drugs on platelet function.⁵⁰² In fact no effect was found for cardiovascular drugs on serum TXB2 levels and plasma TXB2 or plasma BTG levels in this group at rest and over time. Therefore the absence of an effect in the young CHD case group is likely to be real, despite the continuation of cardiovascular drugs in the case group with CHD. In addition, the lack of an association between the PCR and serum TXB2 levels in the young CHD group is also unlikely to be confounded by the continuation of medications, since the relationship was not influenced by the presence or absence of medications in the older CHD group. Furthermore, the significant difference in serum TXB2 levels between the two young study groups is unlikely to be confounded by the continuation of medications. Indeed, if an effect existed it would more likely result in a reduction in the difference found since it is possible that cardio-

vascular medications may reduce serum TXB2 production.⁵⁰²

4.5.4.3 Serum TXB2 production and other measures of platelet function. The serum TXB2 levels were not associated with measures of *in vivo* platelet function in any of the study groups. The patient groups examined were clinically stable and did not have measurable activation of circulating platelets, as assessed by assays of plasma BTG and plasma TXB2.²⁹⁸

The PCR gives a measure of platelet reactivity which is probably a measure of reactivity in response to the passage of blood through the blood collection apparatus.⁵⁸⁴ In the older CHD group there was a weak linear correlation between the ability of platelets to aggregate in whole blood in response to an *ex vivo* exogenous stimulus (blood collection), and the level of serum TXB2 produced *ex vivo* in response to endogenous agonists during whole blood clotting. This older CHD group, with increased platelet aggregate formation (lower PCR), had a greater production of TXB2 in clotted whole blood. This relationship was also present in the normal population group. It would appear that whole blood TXB2 levels provide a quantitative measure of platelet reactivity since maximum TXB2 synthesis during clotting appears to be directly related to the ability of the platelets to form aggregates *ex vivo*. However, in the presence of possible *in vivo* activation of platelets, other factors may be operative since maximum serum TXB2 production has been found to be reduced in such circumstances.⁵⁶⁹ Moreover, platelets which have been degranulated by thrombin *ex vivo* manifest impaired synthesis of TXB2 in response to endogenous thrombin and collagen.⁵⁶⁷ Therefore, if platelets have been partly degranulated *in vivo* by partial activation within the circulation, then *ex vivo* synthesis of TXB2 may be affected. However, even though the assays of plasma TXB2 and BTG may only be crude measures of *in vivo* platelet activation, the lack of an association between serum TXB2 levels and these measures mitigates against such a possibility. Furthermore, if platelets were activated *in vivo*, during or in the absence of thrombin formation, a relationship between whole blood TXB2 and the PCR may not be demonstrable.

4.5.4.4 Serum TXB2 production and blood cells. Serum TXB2 levels in a normal population study group have been reported to be influenced by whole blood platelet numbers and Hc.⁵⁷² In our group of CHD patients, there was a weak positive correlation between the whole blood TXB2 concentration and the platelet count in both CHD groups, but not in the normal group. This relationship was of borderline significance in the study groups, and indicates the importance of other factors in the production of serum TXB2 in these patients. Such factors may not contribute to the level formed in normals, but may reduce the strength of any relationship between serum TXB2 production and platelet count within groups with cardiovascular disease. This finding emphasises the point that the measurement of a parameter of cell function and activation may be less influenced by the total number of cells present than by other sub-cellular components present in association with altered function.

Nevertheless, the results presented do not support the need to make direct arithmetic adjustments to the clotted whole blood TXB2 levels for the different PC, Hb and WCC observed. There was only a weak linear relationship between serum TXB2 and platelet count and Hc.⁵⁷² Furthermore, our observations show that differences

exist between populations and that other blood cell indices influence the level in only one group, namely the CHD patients. Therefore, a simple adjustment of the total serum TXB2 for an arbitrary platelet count value may introduce extra bias, whether for intra- or inter-group comparisons. In the evaluation of relationships between continuous numerical variables or categorical data, within or between groups, it would be preferable to adjust the serum TXB2 value for the platelet count using statistical methods which would take into account the weakness of any association, and when necessary weight adjustments appropriately. When the relationship between the serum TXB2 level and the PCR was adjusted for other relevant variables (viz platelet count, Hb, Hc and WCC) using partial correlations, the strength of the relationship was reduced, indicating a contribution to both the whole blood TXB2 level and the PCR by platelets, red blood cells and white cells. This finding is biologically defensible, since TXA2 can be formed by white cells, and red blood cells can release platelet agonists, such as ADP, particularly if there is any haemolysis during clotting.

The problems of type I statistical errors in studies with relatively small numbers are highlighted in this study. However, the likelihood of this form of error can be reduced by testing or controlling for all realistic confounding factors with any positive association.

4.5.4.5 Influence of other variables on serum TXB2 production. *Other aspects influencing the serum TXB2 production and which have important implications for the results of this study have been evaluated in sections 7.1 and 7.4.* The results of this evaluation demonstrate the small but significant contribution to serum TXB2 levels by factors external to the platelet, in particular the presence of white cells in the clotting blood. In addition, other plasma factors influence the serum TXB2 levels, especially blood glucose levels and specific plasma fatty acids in the normal group. In contrast, in the young CHD group, a major influence on TXB2 production proved to be an intrinsic measure of platelet morphology, namely the mean platelet volume (MPV). This variable, the MPV, appears to have a greater influence in young CHD patients than the whole blood milieu. In normal subjects, the effect of extrinsic variables in the blood are more important. Therefore, *not only can different population groups vary in their capability of producing TXB2, but the factors influencing the level can be qualitatively different.* These observations are further discussed in Chapter 7.

4.5.5 Summary

1. The clinical usefulness of serum TXB2 values remains to be defined, although significant variability between individuals reduces its likely utility for clinical purposes.

2. Our results supported, however, that whole blood TXB2 levels may be of value in the investigation of groups of patients with conditions associated with platelet activation.

3. The findings indicated that when comparing serum TXB2 levels between groups adjustments may be necessary to ensure that variations in the platelet

count and other whole blood cell indices are not contributing to differences observed in the whole blood TXB2 levels.

4. It can be concluded that in patients with stable CHD who have no evidence of measurable *in vivo* platelet activation, that the serum TXB2 is not related to measures of *in vivo* platelet function.

5. It is clear that serum TXB2 production is influenced by both platelet specific factors and environmental factors in the circulation, both molecular and cellular. Specifically, there was a relationship between serum TXB2 levels and platelet morphology (MPV) as well as blood cell indices in the young CHD group. In contrast, in the control group serum TXB2 was related to the degree of platelet reactivity as indicated by the stronger relationship with *in vitro* platelet aggregation, the PCR and plasma BTG levels.

6. The observations provided further evidence for potential variation in serum TXB2 levels in different disease states.

Table 4.1. Patient Exclusion Criteria**Electrocardiographic Criteria:**

1. Left bundle branch block.
2. 2nd/3rd degree heart block, resting heart rate < 50/min
3. Any causes for false positive or negative test.

Underlying Diseases

1. Known or predictable complications to beta-blockers.
2. Congestive heart failure, valvular or congenital heart disease, myocarditis, or Prinzmetal angina.
5. Diabetes mellitus, significant electrolyte, renal, thyroid, hepatic or haematological disorder.
6. Known or detected bleeding diathesis.

Medications

1. Clinical safety for withdrawal of all medications.
2. Any aspirin or drug affecting platelet function.

Table 4.2. Patient Risk Factors and Coronary Artery Scores

<u>Exercise Test:</u>	<u>Positive</u>	<u>Negative</u>
Age (years)	59 +/- 9	50 +/- 9 *
Smoking (pack years)	27 +/- 22	28 +/- 26
Weight (kg)	81 +/- 12	82 +/- 8
Cholesterol (mmol/l)	6.4 +/- 1.3	6.0 +/- 1.0
Triglycerides (mmol/l)	1.96 +/- 0.71	2.02 +/- 1.16
HDL (mmol/l)	1.02 +/- 0.27	0.98 +/- 0.40
mean CAS	2.1 +/- 1.8	2.7 +/- 1.6
mean CSS	3.9 +/- 3.1	6.1 +/- 3.0 **
Left ventricular score	3.1 +/- 1.9	5.7 +/- 2.5 ##
Collateral score	5.9 +/- 2.2	4.4 +/- 2.2

* p=0.025 ** p=0.092 ## p=0.014

CAS=coronary artery score; CSS=coronary stenosis score; HDL=high density lipoprotein cholesterol.

Table 4.3. Platelet Count Ratio

Exercise Test	Positive	Negative
1 pre	0.87 +/-0.12	0.81 +/-0.14
post	0.83 +/-0.09	0.74 +/-0.14 *
2 pre	0.87 +/-0.09	0.79 +/-0.13
post	0.86 +/-0.11	0.80 +/-0.12
3 pre	0.86 +/-0.09	0.82 +/-0.11
post	0.84 +/-0.12	0.81 +/-0.13

means (+/-SD), * p<0.02, pre=before and post=after exercise

Table 4.4. Blood cell indices for the combined groups before and after exercise.

	Test	Before Exercise	After Exercise
PC (10)	1	246 +/- 51	287 +/- 62 *
	2	245 +/- 50	286 +/- 49 *
	3	246 +/- 51	282 +/- 37 *
Hb gm/dl	1	14.5 +/- 1.1	15.4 +/- 1.1 *
	2	14.3 +/- 1.0	15.2 +/- 1.1 *
	3	14.2 +/- 0.9	15.0 +/- 1.0 *
Hc %	1	43 +/- 3	46 +/- 3 *
	2	42 +/- 3	45 +/- 3 *
	3	41 +/- 3	44 +/- 3 *
WCC	1	6089 +/-1608	8500 +/- 2557*
	2	5981 +/-1501	8710 +/- 2414*
	3	5883 +/-1534	9550 +/- 2338*

* p<0.001, PC=platelet count, Hb=haemoglobin, Hc=haematocrit, WCC=total white cell count.

Table 4.5. Correlation coefficients for serum TXB2 and platelet count, PCR, haemoglobin, haematocrit and whole blood WCC.

	PCR	PC	Hb	Hc	WCC
A. Exercise study CHD group for 3 separate measurements:					
1	-0.46 #	0.24	0.19	0.17	0.22
3	-0.41 *	0.27 ^	0.35 *	0.31 *	0.32 *
5	-0.29 ^	0.39 *	0.35 *	0.30 ^	0.27 ^
B. Young CHD group:					
	-0.08	0.43 ##	0.29 *	0.26 *	0.42 ##
C. Young normal group:					
	-0.28 *	0.01	0.18	0.01	-0.03

^p<0.09, *p<0.05, #p<0.01, **p<0.005, ##p<0.001

Table 4.6. Regression analysis with serum TXB2 as the dependent variable and Hb, Hc, WCC, PC and age as the independent variables.

Independent		B	SE B	Beta	Rsq	p
Group	Variable					
Old CHD	WCC	0.72	0.15	0.91	0.82	0.005
Young CHD	PC	0.002	0.0002	0.322	0.18	0.022
	WCC	1.072	0.482	0.303	0.263	0.031

See Table 7.1 and 7.2 for abbreviations.

Table 4.7. Analysis of covariance with serum TXB2 as the dependent variable, and Hb, Hc, PC and WCC as the covariates.

(1) Tests of significance for serum TXB2:

Sources of Variation	Sums of Squares	DF	MS	F	sig F
Within cells	4.51	45	0.10		
Regression	1.80	4	0.45	4.50	0.004
Constant	0.77	1	0.77	7.73	0.008

(2) Correlation between the covariate and the predicted dependent variable (serum TXB2):

Variable	Hb	Hc	WCC	PC
serum TXB2	0.5074	0.4657	0.7628	0.7969

(3) Regression analysis of within cells:

Covariate	B	Beta	signif	lower 95%CI	Upper 95%CI
Hb	0.0109	0.2073	0.491	-0.2049	0.0421
Hc	-0.0009	-0.0479	0.874	-0.0127	0.0108
WCC	0.8716	0.2492	0.084	-0.1233	1.8664
PC	0.0020	0.3283	0.018	0.0004	0.0036

See Table 7.1 and 7.2 for abbreviations.

Table 4.8. Correlation coefficients of the PCR with PC, Hb, Hc and WCC in the older CHD group.

Sample	PC	Hb	Hc	WCC
1	-0.07	-0.46 #	-0.46 #	-0.51 **
3	-0.29	-0.53 **	-0.53 **	-0.17
5	-0.16	-0.33 *	-0.34 *	-0.39 *

^p<0.09, *p<0.05, #p<0.01, **p<0.005, ##p<0.001

Table 4.9. Partial correlation coefficients between whole blood TXB2 and PCR (1), with adjustments for PC (2), Hb (3), Hc (4) and WCC (5) separately, and all combined (6).

	TXB2 (1)	PC (2)	Hb (3)	Hc (4)	WCC (5)	Combined (6)
PCR1	-0.46#	-0.46#	-0.42*	-0.43*	-0.41*	-0.41*
PCR3	-0.49#	-0.46*	-0.40*	-0.42*	-0.48#	-0.41*
PCR5	-0.29^	-0.25^	-0.21	-0.20	-0.21	-0.15

^p<0.09, *p<0.05, #p<0.01, **p<0.005, ##p<0.001

Table 4.10. Linear correlations for CONTROLS and CASES with serum TXB2 and measures of *in vitro* platelet aggregation.

Variable	Control	Case	Variable	Control	Case
LT50ADR	-0.28#	-0.12	RADR	0.17	0.33*
LT50ADP	-0.18	-0.11	RADP	0.34 #	0.13
LT50COL	-0.43**	-0.53##	RCOL	0.33 #	0.09
LAGCOL	-0.57##				

*<0.05 #<0.01 **<0.005 ##<0.001

See Table 7.1 and 7.2 for abbreviations.

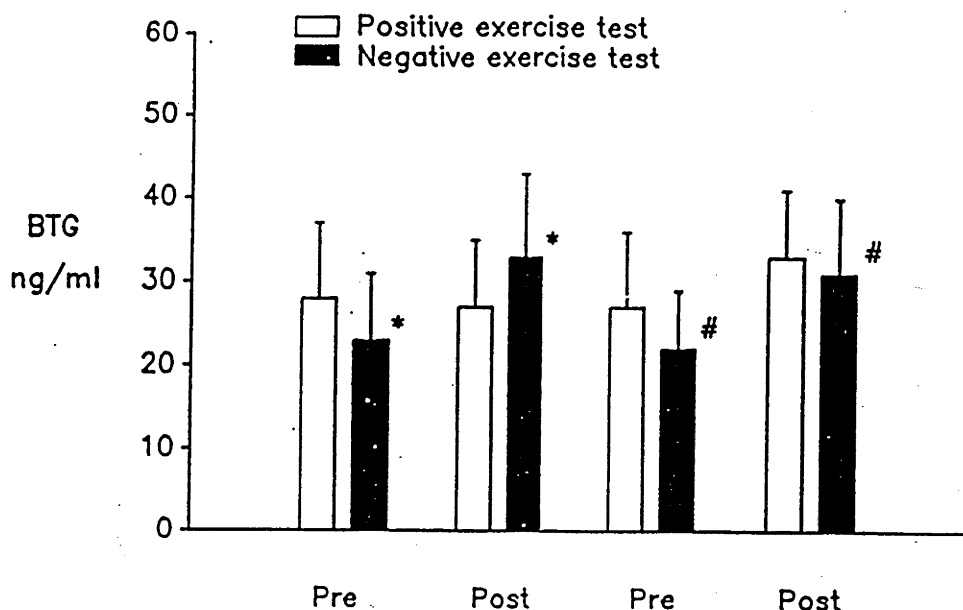


Figure 4.1. BTG (ng/ml) levels before and after exercise in all patients tested (+/-SD), showing a reproducible small but significant increase in patients with negative exercise tests. in the first (* $p < 0.05$), and repeat test (# $p < 0.005$). Pre=pre-exercise value; post=post-exercise value.

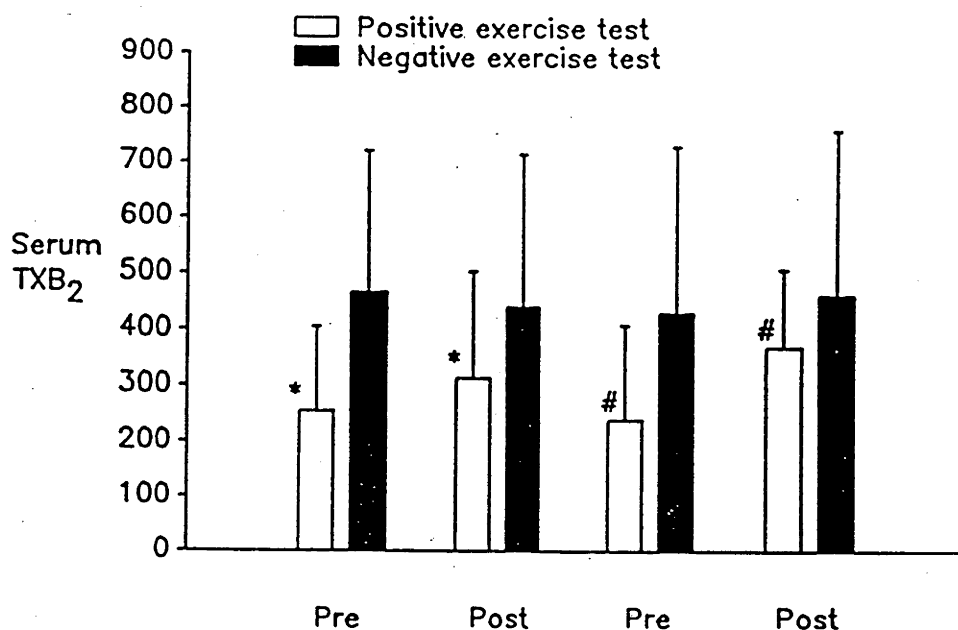


Figure 4.2. Effect of short-term maximum strenuous exercise on serum TXB₂ (+/-SD) in patients with negative and positive exercise tests. A significant difference between the pre and post exercise serum TXB₂ (* $p < 0.005$), was confirmed in the repeat test (# $p = 0.005$) for the group with positive exercise tests only. Pre=pre-exercise value; post=post-exercise value.

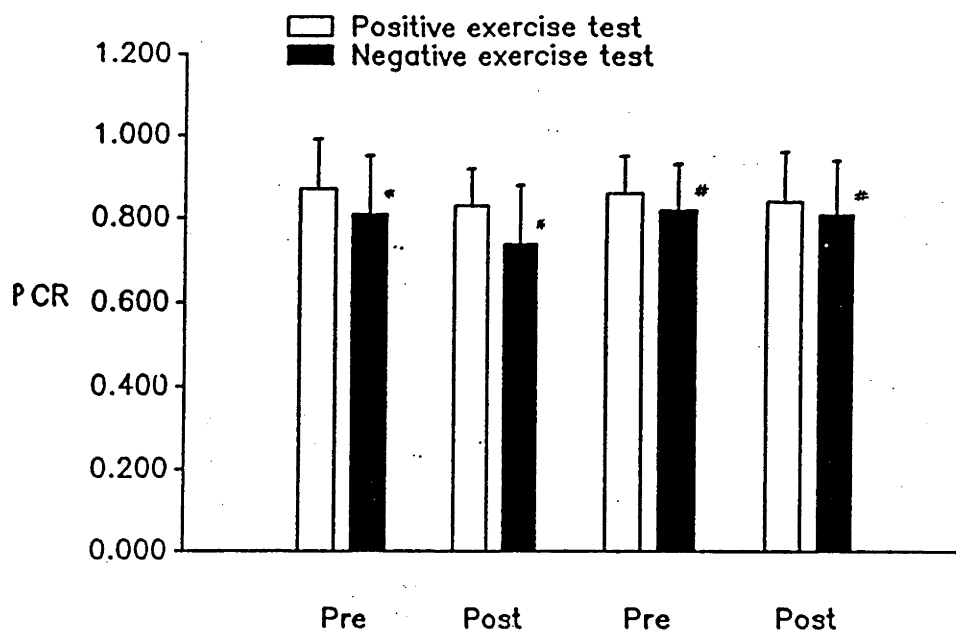


Figure 4.3. PCR before and after exercise (+/-SD), showing a small but significant decrease in the first test for the group with a negative exercise test (* $p < 0.02$), which was not reproducible in the subsequent exercise test (# $p = 0.127$). Pre=pre-exercise value; post=post-exercise value.

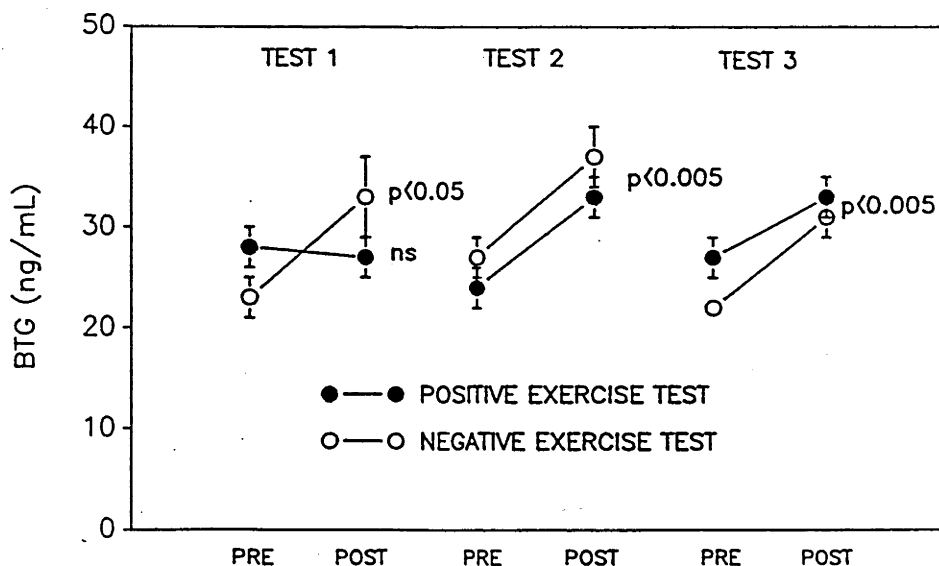


Figure 4.4. Plasma BTG (ng/ml) before (pre) and after (post) exercise in positive and negative exercise test groups, (+/-SEM). Exercise tests 1 and 3 (test 1 and 2) are without medications and exercise test 2 (test 2) is with medications. ns=not significant.

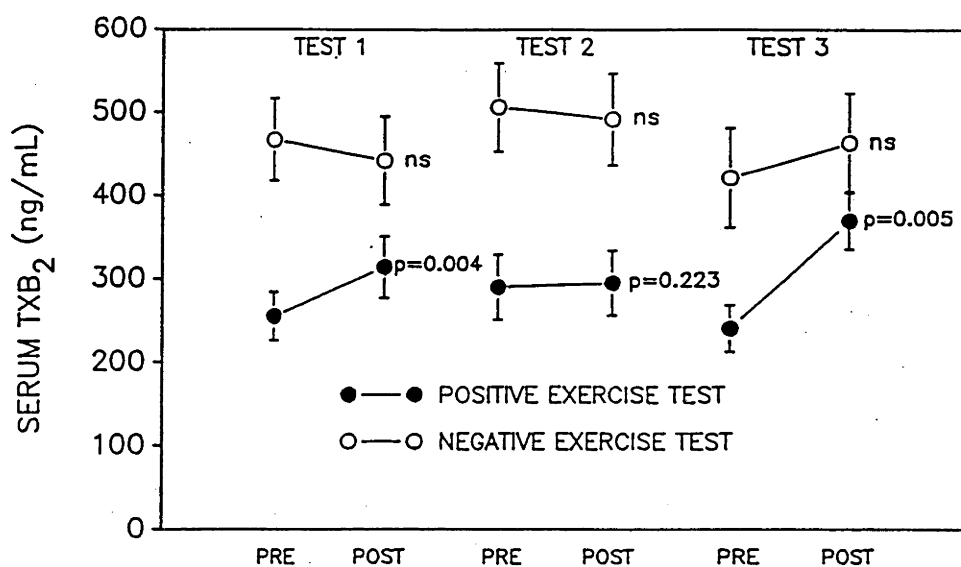


Figure 4.5. Serum TXB₂ (ng/ml) before (pre) and after (post) exercise in positive and negative exercise test groups, (+/-SE). Exercise tests 1 and 3 (Test 1 and 3) are without medications and exercise test 2 is when on medications. ns=not significant.

CHAPTER 5

DIET, LIPOPROTEINS, PLATELET FUNCTION AND CORONARY HEART DISEASE

5.1 RELIABILITY AND VALIDITY OF LIPOPROTEIN MEASURES

5.1.1 Introduction

Reliable and precise measurements of lipoproteins are important for the studies undertaken in this Thesis. The lipoprotein levels used for the studies presented in Chapters 4 and 8 were performed in the Woden Valley Hospital Biochemistry Department, which participates in an external quality control programme and obtains good results for reliability and precision. In the main case-control study (described in Chapter 3) the levels were measured in two laboratories, and the comparison was used to confirm the reliability of the results used for the other studies described in Chapters 4 and 8 of this Thesis. In addition, in this section the validity of the relationship of lipoproteins with angiographically defined CAD is evaluated utilising lipoprotein measurements from the two different laboratories. The results also provide support for the angiographic scoring system used in the studies and described in Chapter 8.

5.1.2 Methods

5.1.2.1 Patients. The case group of young male subjects with CHD used in this evaluation is described in Chapter 3.

5.1.2.2 Blood lipid measurements. The methods for the blood lipid measurements are described in Chapter 2.

5.1.3 Results

5.1.3.1 Cholesterol levels. The cholesterol level of those subjects for whom it was available before or at the time of their diagnosis ($n=27$) was significantly higher than the levels at the time of the study (Table 5.1)

5.1.3.1 Comparison of cholesterol levels. An analysis of variance was performed to compare all the cholesterol levels. The variance between the group means was significantly larger than among the average variance within groups. Thus there are significant differences for the values of the group means ($F=4.7468$, $p=0.0033$). After correcting for multiple comparisons so that the probability was only 5% that any one or more of the comparisons would be significant with $p<0.05$ by chance alone, a significant difference was only found between the prior cholesterol level and the study level (Table 5.2). Small but non significant differences (when corrected for multiple comparisons) were found between the serum cholesterol levels from the study laboratory, the values from the service laboratory and the plasma cholesterol levels from the service laboratory (Table 5.2).

5.1.3.2 Comparison of cholesterol levels. The correlation coefficient between the standard laboratory serum cholesterol and the service laboratory was high, with a correlation coefficient (r) of 0.93, standard error for the equation (SEE) of 2.76, an intercept of 0.10, and slope of 0.77 (SE of the slope=0.04). The same analysis between the standard laboratory serum cholesterol measure and the plasma cholesterol from the service laboratory gave a correlation coefficient of $r=0.90$,

SEE=3.31, intercept=0.18 and slope=0.59 (SE of the slope=0.04). The service laboratory serum measurements were slightly higher and became greater with increasing values, although the differences were small. The plasma values corresponded accurately throughout the measured range but were consistently and modestly higher than the standard laboratory values.

5.1.3.3 Comparison with angiographic scores. The previous cholesterol measurement (for those patients having had the level documented), was inversely related to the number of normal segments, and directly correlated with the extent of disease (CAS, mCAS and pCAS), as well as the number of mild and moderate lesions (25-50% and 50-75%) (Table 5.3). The serum cholesterol level from the standard laboratory had similar relationships, as did the plasma and serum cholesterol from a service laboratory (Table 5.3). The apoprotein B levels had identical or possibly more significant relationships to the extent of disease, the number of mild and moderate lesions and the number of normal segments than did the cholesterol levels (Tables 5.3, 5.4 and 5.5).

Triglyceride levels from both laboratories had no consistent relationships with the extent and severity of disease, although there was a positive association with the number of normal segments (Tables 5.3, 5.4 and 5.5). The HDL cholesterol had no consistent relationship with any of the angiographic measurements (Tables 5.3, 5.4 and 5.5). Equally, apoprotein A1 levels were not related to any of the angiographic measures (Tables 5.3, 5.4 and 5.5). These associations will be further evaluated and discussed in the following section of this Chapter.

5.1.4 Discussion

In this section, the relationship between total serum cholesterol from a research laboratory (used as the standard laboratory) with well established quality control methods, as well as the level in serum and plasma taken from the same blood sample and measured in a service laboratory, also with a well established quality control programme, have been compared. The linear correlation was similar with all measures.

The service laboratory maintains an external and internal quality control programme and has a CV close to 3% with an external standard, a level aimed for with such external standardisation.^{361,585,586} Sustaining both precision and accuracy of measurements by utilisation of external quality controls is the "sine quo non" for the measurement of cholesterol for clinical use.^{585,586} The results demonstrate that measurement of cholesterol by a service laboratory, which maintains good quality control methods, can provide results of sufficient accuracy to compare to the results from the rigorous standards of a standard laboratory, and this included a measurable relationship with CAD. Plasma levels had a slightly higher level which was not statistically significant. Small differences resulting in reduced accuracy of estimating the true mean have previously been noted using various anticoagulants.³⁶¹ However, the reliability would appear to be maintained as the predicted univariate association with CAD is sustained.

A significant difference existed between the previous cholesterol level, measured

prior to the study in those patients for whom it was available, and the measurements taken at the time of the study, often many months after the diagnosis. Numerous confounding factors may contribute to such a difference, such as changes in diet and other sources of clinical variation,⁵⁸⁶ in addition to the effect of regression to the mean of repeated measurements.³⁷⁰ In view of the probability of dietary changes having an effect on the current cholesterol level, the previous measurement would be expected to more accurately reflect premorbid levels, and hence have a relationship with CAD as demonstrated. Indeed, the previous cholesterol, in those patients for whom it was available, appears to have a stronger relationship with the extent of coronary atherosclerosis than the levels measured at the time of the angiographic assessment.

5.1.5 Summary

1. These results demonstrate that cholesterol measurements from a service laboratory with appropriate quality control methods can provide information that is both precise, accurate and relevant to the disease process. The reliability for the clinical use of results from a service laboratory with thorough quality control is well validated.

2. The measurements of cholesterol performed in the Woden Valley Hospital Biochemistry Department are of sufficient reliability and precision to be used as a measure in studies assessing potential confounding factors as described in Chapters 4 and 8.

5.2 THE RELATIONSHIP BETWEEN BLOOD LIPOPROTEINS AND ANGIOGRAPHICALLY DEFINED CORONARY ARTERY DISEASE

5.2.1 Introduction

Numerous epidemiological studies have identified individual attributes associated with an increased risk of developing CHD, especially abnormalities of plasma lipoproteins.^{258,371,587-589} The close relationship between the severity and extent of coronary atherosclerosis and the clinical disease expressed as CHD is beyond dispute. Autopsy studies have demonstrated that elevated serum cholesterol is positively and significantly related to coronary atherosclerotic lesions.¹⁵⁷ However, other risk factors for CHD may be associated with aspects of the processes involved in the development of CHD other than the formation of atherosclerosis.²⁰² Which risk factors are related to the development of the different stages of atherosclerosis requires further evaluation.^{157,202} No single lipid or lipoprotein abnormality has been shown to adequately discriminate between patients with and without CHD and lipid abnormalities have been found to be weak predictors of CAD.^{590,591} Moreover, various lipoproteins appear to have different predictive value for CHD, depending on the age of the subjects and the level of other lipoproteins.⁵⁹²

More recent studies employing multivariate analysis and angiographic scoring methods provide consistent evidence that apoprotein B and LDL cholesterol levels contrib-

ute significantly to the extent of coronary atherosclerosis,^{206,207,591,593} particularly in males with premature CHD.²⁰⁷ In addition, these lipoproteins may also have some influence on disease severity,^{590,593} although possibly not in young males.^{207,592} Other lipoproteins, notably HDL cholesterol and the apoprotein A group of lipoproteins, exert little or no measurable effect on either severity or extent of coronary atherosclerosis.^{206,207,365,592-597}

One study, the original study which used the angiographic scoring system employed in the present report, failed to find any correlation between lipid risk factors and a stenosis severity score.²⁰⁷ However, the study group in that report comprised young males with a recent MI.²⁰⁷ The severity score may, therefore, have been influenced by factors that complicate atherosclerosis, such as thrombosis and plaque rupture, which may be influenced to a lesser degree by lipoproteins, but nevertheless contribute to the progression to severe coronary stenosis. However, in another study of a more heterogeneous population who had not sustained a recent infarct, apoprotein B levels were found to be independently related to a stenosis score.⁵⁹³ These contradictory observations may be due to a selection bias. *Therefore, the aims of the analysis in this section were to:*

1. Evaluate the relationship of the lipoprotein levels with angiographically defined CAD in the study population.

2. Investigate the possibility that a selection bias may explain the presence or absence of an association between cholesterol and apoprotein B levels, and the severity of CAD. This is an important consideration since the progression of coronary artery stenosis may be largely determined by mechanisms other than those which involve lipoprotein abnormalities.^{202,207,593,598} If the lipoprotein risk factor(s) are only related to the extent of coronary atherosclerosis and not to the discrete obstructive lesions, such an observation would support the concept of there being independent factors, possibly thrombogenic, contributing to the risk of obstructive CAD.

5.2.2 Methods

5.2.2.1 Subjects

The study group comprised the 53 young male subjects with CHD described in Chapter 3.

5.2.2.2 Lipoprotein measurements.

The methods and measurements are described in Chapters 2.

5.2.2.3 Coronary angiographic scoring methods.

A detailed description is provided in Chapter 8 and elsewhere.⁶¹

5.2.2.4 Statistical assessment.

The statistical methods used are as outlined in Chapter 2. In addition, lipoprotein levels were categorised into 4 groups with approximately equal numbers of subjects in each group, and the angiographic scores compared between each group. This allowed confirmation of any relationship by using an alternative method of analysis and also if any threshold associations existed which could give a false negative result with assessment by correlation. Significance differences among the mean angiographic scores of these categorised groups was

determined by ANOVA (Statistical Package for Social Sciences ,SPSSX). The Bonferroni t-test was used to determine differences between the means of particular pairs of groups if significant differences were documented, taking into account multiple comparisons.

5.2.3 Results

5.2.3.1 Patient characteristics. A full description of the patient characteristics is detailed in Chapter 3.

5.2.3.2 Correlation between angiographic scores and lipid values. When correlating patient age with the angiographic scores, the older patients in this relatively young group with premature CHD had fewer normal arterial segments as well as more severe and more extensive coronary disease (Tables 5.3, 5.4 and 5.5). The NYHA classification of functional capacity for the occurrence of angina correlated consistently with disease severity, and also with the collateral score and the number of arterial segments supplying collaterals, but to a lesser degree with the coronary atheromatous score (Tables 5.3, 5.4 and 5.5). The collateral score and the number of arterial segments supplying collaterals correlated only with the NYHA functional class for angina, and not with any lipoprotein value (Table 5.5). The left ventricular score was only associated with the number of previous AMI (Table 5.5).

The previous cholesterol measurements for those patients (n=27) having had the level documented was inversely related to the number of normal segments, and directly correlated with the extent of disease (CAS, mCAS and pCAS), as well as the number of mild and moderate lesions (25-50% and 50-75% luminal narrowing) respectively (Table 5.3), as did the current cholesterol level (Table 5.3). The calculated LDL cholesterol was inversely related to the number of normal segments, and was directly related to those scores measuring the extent of disease (CAS and mCAS) and the number of lesions of mild to moderate severity (10-20%, 25-50% and 50-75% reduction in lumen diameter)(Table 5.3). The LDL cholesterol level was not significantly correlated with the severity scores (CSS and mCSS) or the number of severe lesions (75-90%, 90-99% and 100% luminal narrowing)(Table 5.3). The apoprotein B levels had identical and possibly more significant positive relationships to the extent of disease, and the number of mild and moderate lesions, and correlated inversely to the number of normal segments (Table 5.3, 5.4 and 5.5).

Triglyceride levels had no consistent relationships with the extent and severity of disease, although there was an association with the number of normal segments (Tables 5.3 and 5.4). The HDL cholesterol level had no consistent relationship with any of the angiographic measurements for disease severity or extent of arterial wall involvement (Tables 5.3 and 5.4). Apoprotein A1 levels were not related to any of the angiographic measures (Tables 5.3, 5.4 and 5.5).

The associations between the lipoprotein measures and the extent scores were substantiated by categorisation of the lipid variables into 4 groups of approximately equal numbers with increasing values, and comparison of the mean angiographic scores between each lipid category. The results demonstrated a consistent increase in the CAS and the mCAS for the first three incremental categories of apoprotein B

(Figure 5.1). The number of normal segments decreased with increasing incremental categories of the apoprotein B (Figure 5.2). This form of analysis for the categorised lipid variables provides an alternative method which supported the validity of the significant correlations observed. One way analysis of variance (ANOVA) showed that the variances among the group mean of the above angiographic scores were significantly larger than the variance within groups for the subjects categorised according to apoprotein B levels. Significant differences were present between the group means of the CAS and apoprotein B ($F=4.142$, $p=0.0113$), of the mCAS and apoprotein B levels ($F=3.8147$, $p=0.0163$), and between the number of normal segments and apoprotein B levels ($F=2.9591$, $p=0.0425$). An increase in the CAS and mCAS for 4 incremental categories for total cholesterol and LDL cholesterol levels was also observed but the ANOVA did not achieve statistical significance. The mean number of normal segments decreased with increasing incremental categories of total and LDL cholesterol levels. For this angiographic measure the variances between groups was significantly different to that within groups for both total cholesterol (ANOVA, $F=3.2612$, $p=0.030$) and LDL cholesterol levels ($F=3.2612$, $p<0.030$).

The ANOVA was not significant for the categorised apoprotein B levels and the mean severity scores (CSS, mCSS and Jenkins). In addition, the ANOVA was not significant for the other lipid variables (triglyceride, HDL cholesterol and apoprotein A1 levels), when categorised into 4 groups, and the angiographic scores.

The Bonferroni t-test was used to test for significant differences between the mean angiographic scores of particular lipoprotein categories. The Bonferroni p value adjusts for the fact that 6 comparisons are made for the 4 categories. The probability is thus only 5% that any one (or more) of the comparisons were significant at a value of $p<0.05$ by chance alone. For the apoprotein B categorised groups, the mean CAS in the group with the lowest apoprotein B levels was significantly lower, and the mean number of normal segments was greater than in any of the apoprotein B groups with greater levels (Bonferroni $p<0.05$). The mean number of normal segments in the lowest group of total and LDL cholesterol levels, when categorised into the 4 groups, was significantly higher than the third group (Bonferroni $p<0.05$).

5.2.3.3 Multiple regression analysis. Cholesterol, triglyceride, HDL cholesterol, apoprotein A1 and apoprotein B levels were entered into a multiple regression model as independent variables, and each of the cumulative coronary artery scores as dependent variables (Table 5.6). Apoprotein B values maintained an inverse independent association with the number of normal segments, and a positive association with the extent of disease. Age, but none of the lipoprotein values, was independently associated with disease severity (Table 5.6).

5.2.4 Discussion

Coronary artery disease becomes clinically manifested by the intermittent development of severe stenotic lesions associated with plaque rupture and intramural or luminal thrombosis.^{40,41} With this knowledge in mind, our results lend support to the following concepts: (i) that specific lipoprotein abnormalities are risk factors for

coronary atherosclerosis which then predisposes the individual to the clinical expression of CAD; (ii) that the development of severe lesions may relate to factors that complicate atherosclerosis, such as plaque rupture, plaque haemorrhage and luminal thrombosis, which may have different or additional risk factors,^{67,115,238,239,302,599} and (iii) that atherosclerosis may be an intermediate risk factor between CHD, as the clinical expression of CAD, and abnormal lipoprotein metabolism. Nevertheless, the lipoprotein levels are related to moderate lesions which may develop from a rapid accumulation of lipid within the plaque. Also, abnormal accumulation of modified LDL cholesterol may contribute to the destabilisation of an atherosclerotic plaque resulting in plaque rupture, and in this way contribute to the formation of more severe lesions.^{40,41}

5.2.4.1 Cholesterol, LDL and apoprotein B levels and angiographic CAD.

Recently, manual semi-quantitative angiographic scoring systems have been used with the intention of discriminating between atherosclerotic disease involving the arterial wall and the lesions encroaching into the lumen of the artery.^{61,207,593} Such scoring systems have been used to evaluate CHD risk factors with respect to their possible involvement in the atherosclerotic and/or thrombotic component of CHD.⁶¹ In this study we have utilised the more complex of these scoring systems to further examine the relationship between angiographic measures of CAD and the major lipoproteins.

The majority of angiographic studies have demonstrated a positive relationship between total blood cholesterol levels and CAD,^{206,207,220,365,592-594,600,601} although not all studies have shown a correlation,^{596,602} and some studies have only found relationships in younger patients.⁵⁹¹ However, an independent relationship between the various angiographic measures of CAD and total blood cholesterol values is not always maintained using multivariate analysis.^{206,207,590,593}

In contrast, a consistent univariate relationship between apoprotein B and LDL cholesterol levels, and CAD has been well established.^{590,592,594,596,597} An independent relationship is preserved using multivariate analysis.^{206,207,590,593} This relationship may be due to an association with the extent of wall involvement rather than the obstructive severity of atherosclerosis, particularly when such a distinction of angiographic features are separated sufficiently well by appropriate angiographic scoring methods and evaluated by multivariate analysis.^{206,207} Our results are in support of this interpretation, although others have found an independent relationship with stenosis scores thought to measure obstructive severity.^{206,590,593,596} However, the scoring systems used in these latter studies have a greater in-built bias towards the extent of arterial involvement in the stenosis score^{206,590,593,596} than do other methods.²⁰⁷ Multiple minor lesions within a segment of coronary artery is not uncommon in addition to more severe stenoses. Previous studies have included mild lesions in the stenosis score.^{151,590,596} For example, 3 lesions with <50% narrowing in a segment equals one lesion causing 75-90% narrowing in some studies,^{206,593} and in another study 4 lesions with <24% narrowing equals one lesion causing 75-99% narrowing.⁵⁹⁶ The stenosis scoring system devised by Hamsten et al,²⁰⁷ which was used in our evaluation, is less biased towards having the extent of arterial involvement partly included in the severity score by excluding lesions narrowed <25% from this score. Therefore, for the severity score used in this study, if there are

multiple minor lesions within a segment, they are less likely to contribute to the stenosis score. In addition, the severity scoring system of Hamsten et al²⁰⁷ gives a maximum of 16 to a segment, so that in the presence of an occluded segment, minor lesions make no impact on the stenosis score for that segment, decreasing the inherent bias found in other scoring methods which have included mild lesions in a stenosis score meant to reflect the severity of lesions in the coronary arteries.^{206,593,596} For this reason it seems reasonable to conclude that the stenosis scoring system of Hamsten et al²⁰⁷ is more likely to be related to factors such as thrombosis which are involved in the development of severe and/or occluded lesions.⁵⁹³ It was for these reasons, in part, that we selected this method for the current study. Even though the use of a small homogeneous population demonstrated a greater predictive value of specific lipoproteins for the extent of involvement of coronary arteries with atherosclerosis, given the inevitable relationship between minor lesions and severe stenoses, if a larger group had been studied then these same lipoproteins may also have predicted the severity scores as well.

5.2.4.2 HDL cholesterol and Apoprotein A levels and angiographic CAD.

HDL cholesterol and apoprotein A levels are related to the presence of CHD.^{365,595,597,603-606} and angiographic measures.^{365,603,604,606,607} However, as we have found, a relationship between total HDL cholesterol and apoprotein A levels, and angiographic measures of CAD is not always able to be shown.^{206,207,590,592,595,608} Our observations support the proposal that HDL cholesterol and apoprotein A1 levels can be discriminators of disease between groups, but do not correlate with disease severity or extent.^{207,603} It is possible, however, that the relationship is age related, and that there is no relation between HDL cholesterol and apoprotein A1 levels, and angiographically defined CAD in younger males.³⁶⁵ Sullivan and coworkers,⁵⁹³ using a different scoring system, found an independent relationship between HDL cholesterol levels and both a stenosis score and a vessel involvement score in an older population group which included women. Others,²⁰⁷ who have investigated younger male patients with CHD, have found no independent relationship between stenosis scores and HDL cholesterol levels. Previously we have demonstrated an inverse statistical relationship between HDL cholesterol and fibrinogen levels, which remains unexplained.⁶¹ Fibrinogen increases with age, and in the current study population of young males with CHD with a narrow age range, fibrinogen did not correlate with any of the coronary artery scores.⁶⁰⁹ The negative association between HDL cholesterol levels and the severity score observed by Sullivan et al⁵⁹³ may be confounded by an increase in fibrinogen with age in their older population with a greater age spread. Such a potentially confounding influence may well have been controlled for in the present, and in a previous study involving younger age groups with a narrow age range.²⁰⁷ Another important potential reason for a false negative result being present in the same study may arise from the use of total HDL cholesterol measurement.^{604,606} The largest HDL subclass may account for the association between HDL and coronary atherosclerosis.⁶⁰⁴ However, the concentrations of HDL subclasses in plasma probably does not reflect an independent protective mechanism against CAD, since the inverse relation between the plasma level of the largest HDL particles and coronary atherosclerosis is markedly influenced by very low density lipoprotein (VLDL) concentrations.⁶⁰⁴ In patients with a VLDL triglyceride level above 1.65 mmol/l, no relation between disease severity and HDL subclasses can be demonstrated.⁶⁰⁴ In the present study the mean

triglyceride level was 2.44 mmol/l which places the majority of the group into the hypertriglyceride group as defined in Johansson and coworkers study.⁶⁰⁴ Therefore, from the results of the latter study,⁶⁰⁴ no correlation would be expected between CAD and HDL cholesterol levels, particularly when not divided into subgroups. Indeed, the restriction of a relationship to the larger HDL particles,^{604,606} and the influence of triglyceride metabolism⁶⁰⁴ probably accounts for the major discrepancies observed in the literature.

5.2.4.3 Triglyceride levels and angiographic CAD. The triglyceride level had no consistent relationship with either the extent or severity of CAD. Our results fail to clarify the major inconsistencies found in the literature. Several studies have reported a positive univariate relationship between triglyceride levels and angiographic scoring methods,^{592,593,600,601} while others have found no relationship.^{206,207,591,602} The large intra-individual variation and the variability over time in triglyceride levels⁵⁸⁶ may in part explain the variability in the different reports. Most studies obtained blood from fasting subjects in the morning which would help to reduce the variance. Nevertheless, the sources of variation are much greater for triglyceride levels than for other lipid or lipoproteins values.^{361,586,610} Indeed, the intra-individual variation appears to be between 20-40% under normal metabolic conditions.⁵⁸⁶ The magnitude of this variance would not enable any relationships between CAD and the triglyceride levels to be established in study populations of small size. However, the smaller number of homogeneous patients contributes to the reliability of the reported association between CAD and the other lipoproteins with less measurement variance. Furthermore, the sensitivity of the scoring system²⁰⁷ is demonstrated by the results in this present smaller population.

5.2.4.4 Age and angiographic CAD. The positive correlation between age and the extent of atherosclerosis is well documented in the literature.^{207,593} The relationship with disease severity is less consistent, with some studies showing an association,^{591,600-602} while others have not shown any correlation.^{206,207,593} The variability in this relationship may arise from the inevitable bias of the severity score to reflect extent of disease to some degree, as discussed above. In autopsy studies, while large individual variations exist, the frequency of raised atherosclerotic lesions in arteries of males without CHD increases with age.¹⁵⁷ However, there is no simple relationship between the frequency of raised coronary lesions and the onset of CHD.¹⁵⁷ In males with CHD, differences in the averaged extent of raised lesions between age groups is small.¹⁵⁷ Therefore it is not surprising that the available angiographic data regarding the relationship between raised lesions and age remains inconclusive. Nevertheless, in a previous study, using the same scoring system in a larger population group with a broader age range, with multivariate analysis which included the standard risk factors (except apoprotein B), age remained the only independent variable for both the extent and severity of CAD, with the extent score having the stronger relationship.⁶¹

5.2.4.5 Validity of the associations with angiographic CAD scores. The relatively strong relationship between the severity score and the functional class for angina emphasises the validity of the scoring system used in this study, although the limitations of angiographic scoring methods need to be emphasised.⁶¹¹⁻⁶¹³ This observation supports clinical expectation, as does the lack of association with the

extent scores, and also the positive association of functional class for angina with the collateral scores. In addition, the only variable associated with the left ventricular score was the number of AMI, again a predictable observation. Similarly, the negative association between age, cholesterol, and apoprotein B levels, and the number of normal segments also strengthens the reliability of our observations.

In this study, cholesterol measurements performed before the diagnosis had a stronger relationship with the extent of coronary atherosclerosis than the cholesterol measurement undertaken during the study. Numerous confounding factors may contribute to this observation, and these are delineated in section 5.1.4.

There are important limitations to the subjective assessment of angiographically defined CAD.⁶¹¹⁻⁶¹³ There have been significant advances in the quantification of coronary artery stenoses in recent years.⁶¹⁴⁻⁶¹⁸ However, little progress has occurred in finding a suitable quantitative angiographic correlate with the extent of pathological defined CAD to be used in epidemiological studies,⁵⁹³ although valid clinically applicable methods are currently being developed.⁶¹⁹ The traditional method of risk stratification in CAD using coronary angiography has been primarily based on the number of diseased arteries and the left ventricular function.^{620,621} A score expressing the extent of CAD and providing information about minimal or moderate lesions may contribute additional prognostic information beyond the conventional angiographic studies.⁶²¹ The validity of the scoring system used in this study appears acceptable,²⁰⁷ recognising the limitations of the method.^{593,611-613}

5.2.5 Summary

1. In males with premature CHD, lipoprotein abnormalities are associated with atheromatous involvement of the arterial wall, and not with severe lesions encroaching into the lumen. Our study supports previous observations,²⁰⁷ and extends the validity of the relationship to a time frame at least 3 months, or more away from recent unstable CHD.

2. Identification of the clinical, functional and anatomical factors that influence plaque stability have important clinical consequences.⁶²² This concept is one of many that have provided an impetus to determine if thrombogenic risk factors provide a link between CAD and its clinical expression as CHD.

3. The development of generalised atherosclerosis may merely be a contributing factor to the clinical expression of CAD in the form of CHD. The formation of acute severe stenoses may have different risk factors and mechanisms than those for the development of diffuse atherosclerosis.^{4,623}

4. However, the lipoproteins are also related to moderate CAD lesions. Such lesions may also be predisposed to rupture with subsequent thrombus formation. This process may occur without additional factors than rapid accumulation of lipid material in a lesion, in conjunction with alteration in local haemodynamic and shear stress forces, being the cause.

5.3 LIPOPROTEINS AND PLATELET FUNCTION

5.3.1 Hyperlipoproteinaemia and platelet function

A considerable body of evidence exists demonstrating an adverse effect of hyperlipoproteinaemias on platelet function.⁶⁵ Given, the present understanding of their interaction, the alteration in platelet function occurring in the presence of hyperlipoproteinaemia could feasibly contribute to atherogenesis and/or its progression. The changes in platelets that are said to occur in association with elevated lipids could also conceivably give rise to the clinical symptomatic forms of the disease.⁶⁵

The concept of circulating lipoproteins being potentially thrombogenic has long been an area of investigation.^{66,67,624,625} Numerous *in vivo* and *in vitro* methods of assessing platelet function have predominantly, although not universally, shown increased platelet reactivity in association with hyperlipoproteinaemia.^{66,67,625} This interaction appears to be particularly consistent for hypercholesterolaemia.

5.3.1.1 Platelet aggregation. A relationship between increased platelet aggregability and dyslipoproteinaemias has been well established.^{65,66,126,421,423,424,626,627} The available information relating the effect of lipoproteins on platelet sensitivity has been obtained from the investigation of subjects with hyperlipoproteinaemia, which have in general been supported by studies on isolated platelets.⁴²⁰ There may be a threshold level of LDL to effect platelet aggregation, with low LDL concentrations having little influence on aggregation.⁴²⁰ There is an altered response by platelets to aggregating agents following pretreatment with the main lipid density fractions (LDL, VLDL and HDL).^{420,421} Furthermore, a greater effect on *in vitro* platelet aggregation of normal platelets is seen with the lipoprotein fractions from hypercholesterolaemic patients than is seen with the fractions from normolipidaemic controls.⁴¹⁹ Of further interest is the inverse effect of HDL compared to LDL and VLDL.⁴¹⁹⁻⁴²¹ Platelets enriched with cholesterol *ex vivo* are more reactive⁴²² and produce more TXB₂ on aggregation.^{126,128,422}

In vitro studies have in general supported cross-sectional observational studies. Platelet reactivity may be affected by the lipoprotein concentration within a normal population.⁴²³ Hypercholesterolaemic individuals may have enhanced *in vitro* platelet reactivity compared to normal subjects.^{124,424,425} Increased ADP sensitivity in Friedrickson's Type II hyperlipidaemic patients could result in platelet aggregation *in vivo* under conditions in which none would occur in normals. Even more striking is the hypersensitivity of such persons to aggregation by adrenaline. The low concentration of adrenaline required for *in vivo* aggregation in patients with Type II hyperlipidaemia might occur under certain conditions such as severe stress or trauma.⁴²⁴ Such observations have not been universal. In another report, specific *in vitro* platelet aggregation responses to collagen and ADP in hyperlipidemic patients with CHD did not differ significantly from normals. However, there appeared to be some *in vivo* platelet activation in response to ADP and collagen.⁴²⁶ Platelets from patients with type II hyperlipoproteinaemia are more sensitive to aggregation by ADP, adrenaline and collagen;¹²⁴ those from type IV patients did not differ from the normal control group in their responses to ADP and collagen, but they were more sensitive to adrenaline.¹²⁴ Within a population of healthy males, it has been shown

that total and LDL cholesterol concentrations influence the sensitivity of platelets to adrenaline and less so ADP.^{420,423} There was no correlation with HDL or VLDL concentrations, as observed by others.⁶⁷ Platelet hypersensitivity is also induced by cholesterol incorporation into the platelet membrane.¹²⁶ Others, however, have been unable to find any alteration in the sensitivity of platelets to aggregation by ADP, adrenaline and collagen in patients with type II hyperlipidemia,¹²⁷ although the difference may be explained by dissimilarities in laboratory methodology.

5.3.1.2 Cyclooxygenase activity and metabolism. The amount of TXB2 produced after arachidonic acid-induced aggregation of washed platelets may be closely correlated with lipid levels.⁵⁰⁵ These observations support the results of another study which examined thrombin induced production of TXB2 in PRP.⁶²⁸ In that study, increased TXB2 production was found in both type II and IV patients, but more so in the type II patients; the amount of TXB2 correlated with the LDL cholesterol level and the apoprotein B concentration.⁶²⁸ Furthermore, TXB2 production in clotting whole blood was increased, as was the rate of synthesis of TXB2, in type II subjects.⁶²⁸ The amount of TXB2 produced also correlated with the plasma apoprotein B levels in type II subjects.⁶²⁸

5.3.1.3 Platelet release reaction. The release of platelet BTG appears to be increased in patients with hyperlipoproteinaemia and peripheral vascular disease. The total cholesterol level increases with age, as does the LDL cholesterol level within industrialised societies. An increase in plasma BTG levels in old apparently healthy subjects has been reported.^{423,629} Plasma BTG and PF4 levels have been reported to have a strong positive correlation with age, being more pronounced in older females.⁶³⁰ This sex difference has not been reported by others.^{423,629} If there is increased platelet reactivity with age, the actual influence of increasing LDL levels, or, alternatively, the presence of asymptomatic atherosclerosis, remains to be determined.

5.3.1.4 Platelet count ratio. Lowe and his colleagues⁶³¹ studied a group with type II hyperlipoproteinaemia and showed a significant reduction in the PCR in comparison to an age and sex matched control group. However, individuals with clinical arterial disease were included in this study, introducing a susceptibility bias.

5.3.1.5 Possible nature of the platelet-lipoprotein interaction. The presence of a cholesterol enriched milieu may alter the lipid and phospholipid content of the platelet membrane, and hence its function. Shattil and associates used sonicated mixtures of cholesterol and phospholipid (liposomes) to simulate an abnormal environment.¹²⁶ PRP was incubated with the liposomes causing normal platelets to acquire 39% excess cholesterol with no change in the phospholipid or protein content. This was accompanied by a marked increase in platelet sensitivity to aggregation by adrenaline and ADP, without any effect on the response to thrombin or collagen. When platelets were incubated with cholesterol-poor liposomes, there was a 21% reduction in platelet cholesterol, and a marked reduction in their sensitivity to adrenaline.¹²⁶ Incubation with liposomes which caused no change in the cholesterol content, resulted in no alteration in platelet sensitivity to aggregating agents.¹²⁶ Using similar methods others have studied the influence of cholesterol content on the metabolism of arachidonic acid and found a 23% increase in TXB2 release from

cholesterol rich platelets, compared to 14% from cholesterol depleted platelets in the presence of thrombin.¹²⁸ Arachidonic acid and other metabolites were released in significantly higher proportions from cholesterol-rich platelets. The overall increased aggregatory response was associated with an absolute increase in the amount of arachidonic acid released as well as an increase in cyclooxygenase pathway products, with no increase in lipooxygenase pathway products. These findings have been confirmed.⁴²² This latter study also examined the effect of an increased membrane cholesterol to phospholipid ratio on phospholipid metabolism; enhanced hydrolysis of platelet phosphatides in cholesterol-enriched platelets stimulated by thrombin was reported.

It is clear from these and other studies that the lipid composition of platelets may have an influential effect on the function of platelets in man.^{65,423,627}

5.3.2 Methods

The methods for the platelet function studies and lipid measurements are detailed in Chapter 2.

5.3.3 Results

In the control group there was no correlation between any measures of platelet aggregation and blood cholesterol, apoprotein B and triglyceride levels. In the group with CHD, the cholesterol level correlated with the rate of platelet aggregation with adrenaline ($r=0.42$, $p<0.02$) and ADP ($r=0.27$, $p<0.05$), and inversely with LT50% aggregation with adrenaline ($r=-0.27$, $p<0.05$). Apoprotein B only correlated with RADR ($r=0.35$, $p<0.02$).

The PCR and the serum TXB2 level did not correlate with any of the lipid measures in the case or control group. The plasma BTG level correlated inversely with apoprotein A and HDL cholesterol levels in the control group (Table 7.11, Chapter 7). The MPV correlated inversely with the HDL cholesterol level in the case group, as did the triglyceride level (Table 7.1, Chapter 7).

5.3.4 Discussion

The results do not indicate a strong relationship between the lipoprotein values measured and platelet function in normal subjects, but do so for *in vitro* platelet aggregation in the case group. The associations observed are consistent with increased *in vitro* reactivity of platelets to specific aggregating agents (particularly adrenaline) in patients with CHD. A similar relationship was not observed in the control population. Previous studies of normal subjects have demonstrated a relationship between platelet aggregation (particularly adrenaline induced aggregation), and increasing cholesterol levels.^{127,419,422,424,426} A possible explanation for this inconsistency may relate to the lack of individuals with lipoprotein abnormalities in the control group in this study. Most previous studies concentrated on groups with abnormal lipoprotein profiles. The case group on the average had lipoprotein measures differing from the controls (e.g. higher cholesterol levels), and in the group with CHD the level of cholesterol did have a relationship with platelet aggregation,

consistent with previously published results.

5.3.5 Summary

The blood cholesterol level must be included in the multivariate analysis of platelet function and CAD, when appropriate, in the present case-control study because:

1. A potential bias from changes in lipoproteins in the case group due to modification of diet following the diagnosis of the disease exists. There may still be an effect present although diluted by this change and hence not detectable with the small numbers studied.

2. There exists a large but unclear body of evidence implicating a relationship between lipoproteins and abnormal platelet function.

3. There is consistency in the literature and in the present study indicating an influence of the blood cholesterol level on adrenaline-induced platelet aggregation.

5.4 DIETARY ASSESSMENT OF THE STUDY GROUPS

5.4.1 Specific Dietary Areas of Relevance to Platelet Function

5.4.1.1 Abnormal lipoproteins, diet and atherosclerosis. A strong relationship between disorders of lipoprotein metabolism and atherosclerosis is well documented.^{133,258,371,587-589,632,633} An understanding of the exact pathological and metabolic explanation of the association, in terms of cause and effect, remains incomplete.^{67,133,198,383,633-636} Nevertheless, the evidence that elevated blood cholesterol, in particular LDL cholesterol, is essential for the development of arterial atherosclerosis and that the diet of populations and individuals probably plays an important role, is substantial.^{637,638}

The pathogenic mechanisms and cause-effect relationships for the development of CHD have been difficult to define in man. Similarly, the stages in the development of symptomatic CHD have also been difficult to delineate.^{7,381} The present evidence supports the concept of the early development of precursor lesions in youth, with a long "incubation" period prior to the development of the well known clinical syndromes of CHD.^{7,639} The habitual diet of those susceptible to atherosclerosis probably is of major importance in the process.⁶³⁸ For example, dietary induced hypercholesterolemia leads to changes in the endothelium and monocytes, resulting in increased monocyte adherence and migration to the subendothelium; and also monocyte accumulation of lipid to form foam cells in the subendothelium, and migration of smooth muscle cells to the area. These changes have been shown to occur in fatty streak formation.²⁴² This is relevant because the monocyte and the platelet probably interact with the endothelium in the process of atherosclerotic plaque formation.^{7,40,242,295,640} Whether or not the dietary influence is of significance in the non-susceptible individual or those without other CHD risk factors is another issue. Moreover, the diet has other potential adverse effects for those susceptible

which may be independent of the atherogenic properties, for example, in relation to thrombogenic potential (See sections 1.3, 1.4 and 1.5).

5.4.1.2 Habitual diet. The customary diet of a population may well be an essential environmental factor influencing, and even determining a population's susceptibility to CHD.^{633,638} Diet can have an adverse influence on CHD risk in a number of ways. Firstly, as discussed above, abnormalities of lipoprotein metabolism are important risk factors for CHD^{258,641} and are influenced by a number of dietary factors.^{162,637,642} Dietary fatty acids, one of the most important factors determining plasma lipid concentrations,^{258,643} are amongst numerous other dietary factors potentially contributing to the development of CHD.⁶⁴⁴ Secondly, the form and content of a diet not only affects serum lipids but also BP, glucose tolerance and diabetes, central body obesity and other risk factors.⁶⁴⁴ Other important dietary components include dietary cholesterol, total calories, fibre, electrolytes and alcohol.⁶⁴⁴ Finally, the effect of the habitual diet extends to the function of platelets in vitro,^{645,646} including an effect of alcohol on platelet function.¹⁶⁴ That is, the customary diet of an individual or population possibly influences the thrombotic risk associated with atherosclerosis. Indeed, susceptibility to the thrombogenic influences of a diet may be additional to the atherogenic effects, and, as in abnormal lipoprotein metabolism, the susceptibility to environmental influences may be determined by a genetic susceptibility.

5.4.1.3 Dietary fatty acids, plasma lipids and atherosclerosis. Dietary saturated fatty acids have been implicated as an important factor in the production of increased plasma cholesterol in populations with an increased presence and risk of CHD.^{258,643,647,648} The potential influence of dietary factors is supported by a substantial body of literature.^{638,644}

A reduction in dietary saturated fatty acid is the most effective single dietary means of decreasing blood cholesterol levels.⁶⁴⁴ However, not all saturated fatty acids elevate plasma cholesterol^{648,649} and the effect of either saturated or unsaturated fatty acid on plasma LDL cholesterol levels is dependent upon the concentration of cholesterol in the diet.⁶⁴⁸ A diet high in stearic acid significantly reduces plasma levels of cholesterol and LDL cholesterol compared to a diet high in palmitic acid, the reduction being as much as that achieved with a high oleic acid diet.⁶⁴⁹ Increased linoleic acid intake lowers plasma cholesterol although the amounts needed to achieve this are large.⁶⁵⁰ A high intake of polyunsaturated fatty acid and plant protein, and a low intake of animal protein are associated with lower levels of potentially atherogenic lipoproteins.⁶⁵¹ In addition, the balance of evidence available at present indicates that monounsaturated fatty acid potentially have beneficial effects on blood lipids, either reducing LDL cholesterol or increasing HDL cholesterol levels.^{652,653}

The amount of dietary cholesterol is of debatable relevance^{654,655} for the normolipidemic individual.⁶⁵⁵ However, a meta-analysis of the effect of dietary cholesterol indicates that serum cholesterol is increased by added dietary cholesterol.⁶⁵⁶ The magnitude of the change is greatly influenced by the baseline dietary cholesterol in a hyperbolic fashion.⁶⁵⁶ The higher the dietary cholesterol the less the influence on serum cholesterol.⁶⁵⁶ Moreover, despite the modest average effects of dietary cho-

lesterol, some individuals are more responsive and others less responsive.⁶⁵⁶ Cholesterol intake also appears to be related to the smaller LDL particles which are an important atherogenic lipid component.⁶⁵¹

5.4.1.4 Dietary fatty acids and platelet function. A broad body of literature has accumulated evaluating the influence of plasma fatty acid on platelet function, in both *in vitro* and *in vivo* studies. Detailed reviews have been written by a number of authors illustrating the relationship.^{65,67,625,646,657-659}

A high intake of saturated fatty acid is believed to increase the risk of arterial thrombosis.^{65,625} This may be through a number of mechanisms, including increased platelet reactivity which is more affected by saturated fats than by blood lipids.⁶⁴⁵ An apparent opposite effect on platelet reactivity can be achieved by increasing the intake of polyunsaturated fatty acid at the expense of saturated fat.^{645,659} Beneficial effects, as measured by less reactive platelets, of diets rich in polyunsaturated fatty acid are well documented,^{660,661} although the exact effects are not fully elucidated.

5.4.1.5 Dietary fish oils and platelet function. The hypothesis that fish oils may prevent or retard the development of CHD and atherosclerosis has attracted considerable interest but remains a hypothesis.⁶⁶² Nevertheless, the effect of fish oil, and probably more specifically omega-3 fatty acid (n-3 fatty acid) which comprise a dominant component of fish oil, on platelet function has been well documented.⁶⁶² The potential protective effect on atherosclerosis of diets enriched with fish oil has been partially attributed to the influence of the diet on arterial thrombosis.⁶⁶² However, the area at present is in a relative state of uncertainty, so that, even the type of fish consumed may influence platelet function differently.⁶⁶³

5.4.1.6 Dietary salicylates. Interest in the levels of salicylates occurring in food groups has developed because of their potential effect on atopic conditions such as urticaria and asthma.⁶⁶⁴ Most fruits, many vegetables, some herbs and spices and tea contain important sources of food salicylates.⁶⁶⁴ Cereals, meat, fish and dairy products contain none or negligible amounts.⁶⁶⁴ The amount of salicylates ranges from about 10 to 200 mg/day in Western diets.⁶⁶⁴

5.4.1.7 Impact of the above observations on the current study. Despite the lack of scientific and clinical clarity,⁶⁶² the potential benefits of fish enriched, and therefore n-3 supplemented diets, has been widely disseminated in the press to the general public. Similarly, the attempts to change the population's diet has been a prime goal of many National Health Advisory Bodies. This knowledge may clearly influence the diet of those individuals who have developed CHD or have strong risk factors for its development. A cross-sectional observational case-control study undertaken after the diagnosis may therefore be biased and hence confounded by the effects of any changes, conscious or unconscious, in the subjects diet, in particular cholesterol-lowering dietary changes. This latter proposal is supported by the lower blood cholesterol level found in the subjects with CHD compared to their previously known cholesterol level.

5.4.2 Dietary Assessment Methodology

5.4.2.1 Methods available. Three main methods for estimating group and individual consumption of food have been used in epidemiological studies (although they do not constitute the only valid methods). Firstly, a dietary history is directed towards obtaining a qualitative and quantitative assessment of a subject's usual eating pattern. Documentation of a dietary record is undertaken by the subject with the aim of measuring current dietary intake and usually spans 7 days or 20 consecutive meals.⁶⁶⁵ An alternative method is the diet-recall interview which is directed toward detailing food consumption for a specific period, usually 24 hours. Longer periods are subject to defects in memory.⁶⁶⁵ A dietary recall method has been combined with a history of the frequency of the consumption of various foods in order to obtain a more accurate measure of the subject's usual diet.^{666,667} These methods have been evaluated and have been found to have a high degree of correlation between estimates of food consumption and all are suitable for case control studies.⁶⁶⁵ The accurate retrospective measurement of the long-term diet is very difficult and may not reflect the current diet.^{472,668} However, the recall method is the best predictor for estimation of past food and nutrient consumption.⁶⁶⁹ The limitations of the utility of these methods must be acknowledged because of the important influence that a disease itself, and knowledge of the disease, may exert on current food intake.⁶⁶⁵

Ideally the intake of energy and nutrient content of dietary food should be analysed chemically since the energy values and nutrient content of food documented in food tables are only estimates from averages. In group survey work, the cost and time required for chemical analysis makes the method impractical for most clinical and epidemiological research. Assessment of nutrient intake using food tables has become an established method for dietary surveys.⁶⁷⁰

The 24 hr dietary recall method provides reliable estimates of the average intake for groups, and the data has been comparable to that obtained by more time consuming methods.⁶⁶⁸ Nevertheless, the reliability and validity of the 24 hr dietary recall method is reduced when used to characterise individual patterns of food consumption, or identify individuals having some form of nutritional risk.⁶⁷¹ Variation in food intake over seasons of the year, days of the week and work periods are major sources for intra-individual variance for the short recall methods. The recall method is prone to over-reporting low in-takes and under-reporting high intakes with the attendant bias of the "flat-slope syndrome".⁶⁷²

A dietary record covering 7 consecutive days, or 20 consecutive meals, has been recommended as an adequate time to obtain a degree of validity.⁶⁶⁵

5.4.2.2 Methodological limitations. The accuracy of self-report methods of assessing food intake had been questioned and the validity of methods for measuring normal dietary intake have been notoriously difficult.⁶⁶⁸ However, there appears to be an acceptable correlation between measured food intake and a dietary report, and self-reported food intake may be more accurate than previously thought.⁶⁶⁷

Reliability problems arise when an individual's "usual" intake has sources of variability that cannot be attributed to true differences between individuals.⁶⁷³ Such error

can arise for many reasons,⁶⁷³ as discussed above. The basic assumption that an habitual diet exists may be incorrect. There may be inherent errors within food composition tables. Recall accuracy of food consumed may be inaccurate. Interview variability is a well documented area of bias. A training effect may occur with repeated interviews or diary entries. There may be differences in absolute nutrient content for non-generically named foods. The day of the week effect due to day to day variation can be a substantial confounding factor. Work related differences and data handling errors add to these more commonly encountered sources for introduction of bias described above.^{666,671,673} Food composition tables are useful and have a well recognised utility, although their limitations are conspicuous.^{666,668}

5.4.2.3 Food measurements and estimations. Food diaries can accurately indicate the food eaten by an individual at a given time, if the subject is properly motivated and is able to record carefully the intake with appropriate instruction beforehand and checking of the record by interview afterwards.⁶⁷⁴ However, comparison of estimated food portions with actual measurements have revealed potentially large errors in portion measurements,⁶⁶⁸ although others have found an acceptably reliable agreement.⁶⁶⁷ Conducting a dietary survey requiring the subject to measure the quantities of foods means creating new practices in the kitchen and at the table which may introduce bias in the form of modification of the "normal" diet. An individual's food intake can be affected by the record-keeping procedure itself.⁴⁷²

An estimate of nutrients of special interest can also be difficult. For example, the fat content may vary between meat cuts and fat may be added or lost during cooking. However, when fat intake is assessed by a dietary history, the correlation between repeated measures is high for the same observer, but low for different observers.⁴⁷²

The contribution of systematic errors, such as coding, to the total error of calculated intakes for a seven-day period is likely to be small for persons with variable eating patterns.⁶⁷⁵ However, it still will be necessary to standardise the procedures and the coding, preferably by using the same interviewer, to reduce variance associated with subjective interpretation, thereby increasing the reproducibility of the calculated intakes.⁶⁷⁵

5.4.2.4 Methods of statistical inference. Correlation between dietary information and measured parameters will be made with the knowledge that intra-individual variation and random errors of measurement may lead to large confidence intervals, misclassification and reduced accuracy of correlation coefficients. These effects can reduce the power of statistical inference and produce false negative correlations.⁶⁷¹ In such circumstances, the interpretation of any observation, in particular negative results, must be considered with caution in the absence of a highly reliable and validated estimate. However, if intra-individual variation and measurement error are random, there will be no bias in the mean value.⁶⁶⁸ Comparison of the results of dietary survey measures of samples from specific populations can be performed, assuming the variations are random about the mean, by comparing the means in the upper and lower quintiles of the characteristic being considered.⁶⁶⁸

Dietary survey methods may show differences in the averages among samples of people when there are large differences in the diet at the time of the survey.⁶⁶⁸ However, small or inconstant differences are unlikely to be detected. Moreover,

classification of an individual with respect to their diet, or specific nutrient, may give rise to major methodological limitations. For the dietary survey methods, the more important areas of bias include the misclassification resulting from wide intra-individual variation, instability or inconstancy of the diet over time, such as in seasonal variation within the distribution of the population over longer periods of time.⁶⁶⁸ As a result of these and other causes of error described above, the intra-individual variation may be as large as the inter-individual variation.⁶⁶⁸ If the nutrient content is reported in proportion to total energy consumption, total variance tends to fall, inter-individual variance falls, and intra-individual variance also improves, but to a lesser degree.⁶⁷³

5.4.3 Methods

5.4.3.1 Rational for the methodology employed. Since, within a homogeneous population, the variation for each individual for any particular dietary factor may be as large or larger than inter-individual variations,⁶⁷¹ a seven day dietary diary was obtained. This approach was undertaken to improve the estimate of the true mean intake for any dietary factor for the individual, thereby increasing the ability to distinguish group differences for homogeneous study populations.^{671,668} Indeed, the methods described above are all applicable to case-control studies. The diet history was selected since the current food intake may be influenced by the disease and/or knowledge of the diagnosis.⁶⁶⁵ The main purpose of the dietary assessment was to determine if any major differences existed between the case and control group since dietary differences have the potential to influence the measurement of a number of CHD risk factors.^{150,469,636,651} as well as some of the platelet function measures.^{663,676} Assessing specific areas of possible differences between the two groups is therefore necessary to determine if areas of bias have arisen from differences in specific dietary factors, particularly if affecting platelet function parameters.

The main value of the method used will be to determine differences in grouped sample averages. The observations will need to be viewed with caution, particularly because of the potential for false negative results.

5.4.3.2 Methodology. The nutritional information obtained was the content of the subject's diet for the 7 days before the blood sampling. The diet was assessed as a food group analysis. The subject estimated the amount of food by describing the number of portions of specific foods and food groups. This was estimated by the subject with the assistance of photographs of food models and descriptive measures from food tables. All data on nutrient composition of foods for approximated portions was obtained from published sources although the comparison was made only with the groups of food types and the number of portions in that group.

The subjects were carefully instructed about the methods of documentation, importance of continuing their normal diet, on the use of the food model descriptions and photographs, and of what a portion of any specific and commonly used food consisted. The initial instructions on how to keep an accurate diary of all food and beverage consumed at each meal and between meals were given to the subjects by the same individual (the author). The food diary was documented on a single subdivided sheet and on a daily sequential basis. The week consisted of meals which were routine and

standard for the subject and his family. A follow-up review of the recorded intake with the participants was undertaken in order to check for accuracy and completeness. The follow-up assessment was also carried out by the same interviewer.

The dietary diaries were then coded by another person to blind the author to the identity of the subject for all the diaries. These were randomly coded and mixed so that it was unknown to the author whether the subject was from the case or control group. The number of portions of specific foods and groups were documented and are outlined in Tables 5.7, 5.8 and 5.9.

5.4.4 Results

The number of portions of meat taken by the case group was lower than the control group with a borderline level of significance (Tables 5.7 and 5.9). There was no difference in the number of portions for individual types of meat, that is, beef, chicken, pork and lamb. Nor were there any significant difference in the way the meals were prepared, whether fried, grilled, baked or in casserole form. In particular, no difference was observed for fried foods (Table 5.8). The case group tended to have more fish in a baked, grilled or casseroled form, although this was not statistically significant because of the high inter-individual differences leading to the high variances for the groups (Table 5.7). The case group consumed significantly less full cream milk, substituting low fat milk (Table 5.9). In addition, the case group consumed significantly less sweet foods high in fat, such as pastries, cakes, sweet pies and so on, than the controls (Table 5.9). Therefore, the total fatty food intake in the control group was greater than in the case group.

Of similar importance, the case group consumed significantly more fruit than the control group (Table 5.9).

Because the relevance of alcohol to platelet function, this is examined in more detail in the next section.

5.4.5 Discussion

The cholesterol level for those with a previous measurement was significantly greater than the level measured at the time of the study in the case group (Table 5.1). It would be reasonable to assume that the diet for the case group had been modified since the diagnosis. To confirm such an assumption it would be necessary to extend the dietary assessment by obtaining a previous and current food group recall estimation. This was not performed as the main purpose of the food intake evaluation was to determine if there were, in the current diet, any differences which might have influenced platelet function, given the potential differences in diet after a diagnosis and the possible influence of that change on platelet function. The potential for such an effect is highlighted in the introduction of this section which illustrates some clear examples where this could occur. For instance, an altered saturated fat, or other fatty acid intake, has the potential to measurably influence platelet function.

The presence of a lower intake of food with greater saturated fat and cholesterol content in the case group could potentially result in reduced platelet reactivity and

aggregation. Such an effect could lead to a false negative result when comparing the case and control group, or reduce any differences found. Therefore, any measures indicating increased platelet reactivity or aggregability in the case group may be less than at the time of, or before the diagnosis of CHD was established. This dilution bias is unavoidable in the current study because of the case-control design, and the limitation has to be fully recognised and taken into account when interpreting the primary study results.

Another important bias that has not been controlled for in this evaluation is response bias. The presence of acquiescence, or socially desirable responses, by individuals undertaking self-response questionnaires is well documented.^{471,677,678} Ideally, some form of assessment of this potential bias should be administered simultaneously as any other self-response inventories.^{677,678} The lack of such an assessment does pose a problem in interpretation of the results. However, the internal consistency with regard to the reduction of the total blood cholesterol levels since the time of diagnosis in the case group does support a real change in eating behaviour as documented. Similarly, the "halo-effect" would also influence the control group, particularly in regards to alcohol intake.

5.4.6 Summary

1. The subjects in the group with CHD, on the average, appear to eat less fatty foods, less dairy products and more fruit than the control group. There was also a reduction in the contemporary cholesterol level compared to previous levels in the same group, suggesting that a change in habitual diet has occurred since the diagnosis.

2. The influence of the dietary differences could have lead to reduced platelet reactivity and measures of aggregation in the case group compared to the control group.

5.5 ALCOHOL, PLATELETS AND CORONARY HEART DISEASE

5.5.1 Introduction

5.5.1.1 Alcohol and cardiovascular mortality. An inverse association between alcohol consumption and the risk of CHD has been documented in autopsy, case-control, geographic and other types of cohort studies.⁶⁷⁹ These cohort studies have demonstrated that people who regularly consume small amounts of alcohol are at lower risk of CHD than abstainers.⁶⁸⁰⁻⁶⁸⁶ Indeed, evidence supporting the hypothesis that moderate alcohol use reduces the risk of CHD obtained from numerous prospective studies.^{679,681,686,687} Some prospective studies, however, have shown no relationship between alcohol and CHD when other risk factors are taken into account.⁶⁸⁴

The mortality associations for alcohol intake may follow a U-shaped curve.^{681,687,688} Cardiovascular and total mortality rates appear to be greater in non-drinkers and in heavier drinkers than light to moderate drinkers.^{681,687} Nevertheless, even at low levels of alcohol intake the risk of death from cancer or stroke was greater than in

nondrinkers.⁶⁸⁷

The apparent protection against CHD of light daily alcohol intake may be a consequence of methodological factors in the design of these studies. Firstly, an increased incidence of desirable attributes may be present in the mild to moderate alcohol intake group. A group of light daily drinkers who have the lowest incidence of CHD had the lowest mean BP and BMI, and contained the lowest proportion of smokers and manual workers.⁶⁸⁴ Secondly, it is also possible that the observed alcohol-mortality relationships in prospective studies are produced by symptoms and disease present at the time of screening, and by the prior movement of subjects into non-drinking or occasional drinking categories.⁶⁸⁹ In the British Regional Heart Study the alcohol-mortality relationships for total and cardiovascular mortality were only present in men with cardiovascular disease or cardiovascular-related doctor diagnosed illnesses at the initial evaluation.⁶⁸⁹ In the Honolulu Heart Study the incidence of CHD in ex-drinkers was almost twice that seen in current drinkers.⁶⁸⁷ The Western Electric study demonstrated a small decreasing trend in CHD mortality with mild to moderate alcohol use.⁶⁹⁰ This trend, however, was not statistically significant when differences in BP and cigarette smoking were taken into account.⁶⁸⁹

The majority of prospective studies relating alcohol intake to cardiovascular disease, CHD and total mortality, consistently demonstrate higher total and cardiovascular mortality rates for non-drinkers and heavy drinkers than light or moderate drinkers.⁶⁸⁹ However, the apparent protective effect of alcohol may not be due to the moderate use of alcohol, but instead the downward drift from heavy drinking towards non-drinking under the influence of accumulating ill-health.⁶⁸⁹ Other investigators have argued that this is not the case and that the increase in mortality in non-drinkers over moderate drinkers is not due to preexisting disease or differences in dietary habits.⁶⁷⁹ A recent prospective study supports this argument.⁶⁸⁶

5.5.1.2 Potential mechanisms for protection by alcohol against CHD. The effect on moderate alcohol consumption of lipoprotein and apolipoprotein metabolism offers a biologically plausible mechanism for the apparent protective association of alcohol.⁶⁷⁹ Moderate alcohol intake raises HDL cholesterol,^{684,686,691,692} and this inverse association between HDL cholesterol and alcohol intake has a dose-response relation.⁶⁸⁴ A full review of this association has previously been published.⁶⁷⁹ Serum cholesterol does not show a consistent relationship to alcohol intake,^{684,692} but serum triglyceride is positively associated.⁶⁷⁹ The inverse association between alcohol and CAD does not differ by age or smoking status.⁶⁸⁶

5.5.2 Methods

In the interview with the case and control subjects, each was asked the approximate year that alcohol use commenced, the year ceased if this was the case, the number of days per week alcohol was taken, and the usual number of portions per day when used. The self-reported number of portions were the number of standard glasses of beers, standard glasses of wines and of spirits. The standard glass of beer was defined in the customary terms of those that drink beer, that is, as the equivalent to one 10 oz glass (or one "middie" of beer). From this information, the duration of alcohol intake for those using or having used alcohol, the number of portions per week, the

percent ceasing alcohol use and when, and the percent of alcohol users was documented.

Similarly, in the dietary diary, the subjects were requested to document daily the equivalent to the number of standard glasses of alcohol used for each type of alcohol as described above. The mean number of portions of alcohol used by the groups and the number of individuals using alcohol at the time of the assessment was thus documented.

The estimated amount of alcohol from the questionnaire and the dietary diary, the intake for the two groups and the percent and number of former and current drinkers were compared.

5.5.3 Results

5.5.3.1 Group differences. The difference in the estimated number of portions of alcohol previously used by both the case and control groups was not significant (Table 5.10). In the case group, the estimate was slightly higher than in the controls, but because of the wide variance the difference was not significant (Table 5.10). The documented number of portions of alcohol in the dietary diary was slightly greater in the control group, but, again, the difference was not significant given the large standard deviation (Table 5.10). There was no difference in the percent of subjects in the two groups who previously used alcohol (Table 5.11). The length of time that individuals used alcohol in the two groups was also the same. The mean number of years of drinking for the control and case groups was 22.4 ± 10.3 and 21.5 ± 11.3 years respectively (95% confidence interval for the mean difference was -5.2 to 3.4, $p=0.66$).

The number of subjects not using alcohol within 24 hr of the platelet function tests was similar in both groups (35 in the case group and 31 in the control group). The amount of alcohol used by the remaining subjects was also the same (Table 5.10).

However, significantly fewer case subjects admitted to the use of alcohol after the diagnosis of their CHD (Tables 5.11 and 5.12). Approximately 18% of subjects using alcohol in the case group stopped at the time of their diagnosis (Table 5.12). There was a borderline significant difference between the number previously using alcohol and the number at the time of the interview for the case group (Table 5.12). The number not using alcohol was also confirmed by the dietary diary (Table 5.13). In addition, the number of controls using alcohol at the time of the dietary diary was less than the number who stated that they used alcohol at the time of the interview (Table 5.12).

5.5.3.2 Correlation of weekly alcohol intake and other variables

(i) *Coronary heart disease risk factors.* The amount of alcohol used per week correlated positively with the triglyceride level in both the case and control group (Table 5.14). The total cholesterol level was significantly and positively associated with the alcohol intake in the control group (Table 5.14). HDL cholesterol values did not correlate with this measure of alcohol intake. The alcohol intake

correlated with the level of cholesterol and LDL cholesterol in the control group (Table 5.14).

The control group also demonstrated a relationship between alcohol intake and previous cigarette intake (Table 5.14). However, there were very few current smokers in both groups (n=23 past smokers in the control group, n=40 past smokers in the case group, n=4 currently smoking in the case group, and n=7 currently smoking in the control group). None of the other measurable risk factors, including the BP, BMI, and the fibrinogen level, was associated with alcohol intake.

(ii) *Platelet function.* No platelet function measurement correlated with alcohol intake in this study in the control group. There was an equivocal relationship with platelet measures in the case group. The estimated intake of alcohol negatively correlated with the MPV and positively with the PCR (Table 5.14). No other measure of platelet function was related.

(iii) *Alcohol intake and other parameters.* In the control group only, the amount of alcohol intake correlated positively with the MCV, the pack years of cigarettes and the blood urate level (Table 5.14).

(iv) *Alcohol intake and angiographic CAD.* The alcohol intake did not correlate with any angiographic measure of CAD in the case group.

5.5.4 Discussion

These observations demonstrate that the use of alcohol was frequent in both the case and control groups, but there was no major difference between the two groups. There was, however, an increased likelihood that the subjects with CHD stopped drinking alcohol after their diagnosis. Importantly, there was not a significant difference in the percent of subjects using alcohol at the time of the evaluation and when the blood was collected for the platelet function tests and measurement of risk factors for CHD.

The results from this small case-control study confirm that the amount of alcohol use can be estimated reasonably well by a single self-report of intake.⁶⁹³ In the control group, the mean weekly intake estimated by the dietary record was slightly but not significantly higher than estimated in the questionnaire, an observation also reported by others.⁶⁹³ However, the lower number of control group subjects using alcohol during the week of the dietary diary indicates that a single measure of alcohol may not reflect the pattern of drinking for a group. Alternatively, this reduction may arise from intentional under-reporting, although under-reporting would be expected to be less likely for moderate consumption.⁶⁹³ As in other aspects of the dietary diary and self-reported measures discussed above, an important bias that has not been controlled for in this evaluation is response bias.^{471,677,678}

The pattern of alcohol intake by the CHD case group in this study supports the concept that, at least in part, the increased CHD mortality differences related to alcohol intake are due to the downward shift of intake by individuals already with disease symptoms.⁶⁸⁹ As a cross-sectional study, the results cannot negate the ob-

servations from a large prospective study, that, even taking the downward shift into account, there remains an inverse association between alcohol intake and CHD.⁶⁸⁶ However, there are a number of other important observations arising from this latter study. Firstly, despite an increase in dietary risk factors for CHD in the groups with higher alcohol intake, the CHD mortality was less, implying that alcohol can protect against the adverse effects of other dietary risk factors. Those drinking more alcohol on the average also smoked more and had a more frequent diagnosis of hypertension, and despite both of these being very powerful risk factors for CHD, there was less cardiovascular events in the follow up period.⁶⁸⁶ If the observations are correct, alcohol would have a very powerful protective effect against CHD. Secondly, the results confirm that there is no increased risk of CHD in abstainers, although the total mortality data were not reported.⁶⁸⁶ This observation does support the prospect that movement of higher risk individuals into the group of abstainers from alcohol, but not the light to moderate drinking group, may have been a cause of the U-shaped curve for cardiovascular mortality in previous studies.⁶⁸⁹ The exclusion of non-drinkers and men with disorders potentially related to CHD which may have lead to a prior reduction in alcohol intake, did not affect the relative risk associated with alcohol. Thirdly, it would appear that the more an individual drinks alcohol and the more often, then the lower the risk of CHD. Obviously, this probably would be offset by the total mortality increase well known with high levels of alcohol intake, although the data was not presented. From this study, and looking at alcohol intake alone, alcohol appears to impart a strong protective effect against cardiovascular disease.⁶⁸⁶

As in all such epidemiological studies, potential areas of bias exist. Firstly, the group consisted of upper socioeconomic and educated individuals, a population different to that in previous studies.⁶⁸⁹ This is pertinent since both the risk and incidence of CHD is greater in the lower socioeconomic groups,⁶⁴⁶ the prevalence of risk factors are greater^{459,460,646} and the prevalence of increased alcohol intake is greater.⁶⁴⁶ Secondly, the results appear to differ from most other prospective studies. Almost all prospective studies in heavy drinkers have consistently shown an association between increased mortality from CAD and increasing alcohol intake, in contrast to moderate alcohol intake.⁶⁷⁹ Again, there are potential confounding issues leading to such spurious results,⁶⁷⁹ although the observations appear reproducible in other studies.⁶⁸⁹ Thirdly, the groups with higher alcohol intake also used more aspirin on a regular basis, a potentially powerful treatment bias considering acute coronary events are included in the outcome analysis.^{100,103,105,694-698}

Using simple linear correlation without adjustment for other risk factors, there was no association between HDL cholesterol values and prior total weekly alcohol intake in both study groups. However, with greater numbers there may be an association, as has been demonstrated in large studies.⁶⁹³ There was an apparent association between alcohol intake and triglyceride levels and measures related to LDL cholesterol. Others have demonstrated a decrease in LDL cholesterol with alcohol intake,⁶⁹⁹ while other studies have shown no relationship.⁶⁸⁰ It is clear that those who consume more alcohol have a diet richer in fat.⁶⁸⁶ There is evidence that the case group in this study changed both the fat content and alcohol content of their diet, which would have major confounding influence on any associations that may have been present pre-morbidly. This is also supported by the consistent relationship in the controls

between variables that are known to be influenced by alcohol intake. Those that used more alcohol in the control group had a higher MCV, smoked more cigarettes and had a higher blood urate level. The increased use of tobacco in those using more alcohol is well documented.⁶⁸⁶

A number of causes of this possible protective effect of alcohol have been postulated,^{679,699} in particular an elevation of the HDL cholesterol level.⁶⁰⁴ If increased HDL cholesterol does provide a mechanism by which alcohol use can protect against the development of CHD, this would only explain about 50% of the protective effect.⁶⁹⁹ It has been postulated that factors influencing haemostasis are potential candidates for the added protective effect.^{164,699,700} Platelet aggregation in response to ADP appears to be inhibited by alcohol intake,⁷⁰⁰ and by the same level of alcohol use that protects against CHD.¹⁶⁴ There was no measurable association between alcohol intake and platelet function measures, including measures of platelet aggregation in the control group in this study. This may again be due to a type II statistical error, but even if so, the clinical importance of such an association would only be small. However, in the control group, increasing alcohol intake was associated with smaller platelet volume and also less platelet aggregate formation during blood collection. This equivocal association in the case group would in fact increase the possibility of a false negative result because of alcohol use, that is a dilution bias. Nevertheless, a difference in these parameters was documented and moreover, there was no measurable difference between the alcohol intake of the two groups.

5.5.5 Summary

1. Any associations, or lack of association, between alcohol intake and other parameters needs to be interpreted with caution given the major confounding influence resulting from the change in diet, including alcohol use, in the case group since their diagnosis was first established.

2. The differences in platelet function demonstrated in this study are unlikely to be biased by alcohol use resulting in increased platelet reactivity in the case group.

Table 5.1. Lipoprotein measures for the case group patients.

Mean+/-SD		Mean+/-SD	
Cholesterol(Pr)	6.08+/- 1.41	Cholesterol(St)	5.18+/-0.74
Cholesterol(Sr)	5.64+/- 1.11	Cholesterol (Sm)	5.46+/-0.90
Triglyceride(St)	2.21+/- 0.23	Triglyceride(Sr)	2.24+/-0.17
HDL(St)	0.86+/- 0.21	HDL(Sr)	0.95+/-0.23
Apoprotein B	0.95+/- 0.17	Apoprotein A1	1.21+/-0.28

Pr=previous (n=27),
 St=reference laboratory (serum),
 Sr=service laboratory (plasma),
 Sm=service laboratory (serum),
 HDL=HDL cholesterol.
 All measures in mmoles/l.

Table 5.2. Analysis of variance of the comparison between all the cholesterol levels.

Comparison		mean	uncorrected	Bonferroni
		<u>difference</u>	<u>p value</u>	<u>p value</u>
A versus	B	0.90	0.0006	p<0.01 **
A "	C	0.44	0.0825	p>0.05
A "	D	0.62	0.0154	p>0.05
B "	C	-0.46	0.0199	p>0.05
B "	D	-0.28	0.0153	p>0.05
C "	D	0.20	0.3568	p>0.05

A = previous cholesterol (Pr, n=27),
 B = study serum cholesterol (St, from reference laboratory in serum),
 C = service laboratory plasma cholesterol (Sr),
 D = service laboratory serum cholesterol (Sm).

Table 5.3. Correlations between lipoproteins and angiographically defined atherosclerotic disease.

	CAS	mCAS	pCAS	dist	50/75%	25/50%	10/25%
DOB (year)	-0.27*	-0.29*	-0.20	-0.19	-0.31*	-0.10	-0.12
NYHA FC	0.20	0.23	0.40#	0.13	-0.09	0.19	0.03
Chol (Pr)	0.46#	0.44#	0.48*	0.51#	0.35*	0.45#	0.23
Chol (St)	0.23*	0.27*	0.15	0.40*	0.28*	0.25*	0.29*
Chol (Sr)	0.29*	0.33#	0.29*	0.08	0.36#	0.30*	0.33#
Chol (Sm)	0.26*	0.28*	0.15	0.16	0.28*	0.27*	0.29*
Trig (St)	-0.17	-0.20	-0.18	-0.19	-0.26*	0.07	0.02
Trig (Sr)	-0.19	-0.21	-0.19	-0.23	-0.15	0.01	-0.11
HDL (St)	0.01	0.03	-0.10	0.01	0.03	-0.03	-0.04
HDL (Sr)	-0.05	-0.02	-0.16	0.08	0.09	-0.05	-0.01
Apo B	0.35#	0.35*	0.23	0.26*	0.30*	0.33*	0.36#
Apo A1	0.09	0.12	0.02	0.01	0.13	0.09	0.08

*p<0.05, #p<0.01, All values are the Pearson correlation coefficient.

CSS=coronary stenosis score, mCSS=mean CSS, pCSS=proximal CSS, CAS=coronary atherosclerotic score, mCAS=mean CAS, pCAS=proximal CAS, Jenkins=Jenkins coronary score, and LVS=left ventricular score. Data are shown as mean +/- SD. See the Methods section for an explanation of the scores.

Chol=cholesterol, trig=triglyceride, HDL=HDL cholesterol, Apo B=apoprotein B, Apo A1=apoprotein A1, NYHA FC=New York Heart Association functional class for angina. See Table 5.1 for other abbreviations.

Table 5.4. Correlations between lipoproteins and angiographically defined measures of disease severity.

	CSS	mCSS	pCSS	100%	90/99%	75/90%	Jenkins
DOB (year)	-0.25*	-0.25*	-0.22	0.01	-0.34#	-0.17	0.28*
NYHA FC	0.31*	0.34#	0.55**	0.41#	0.13	0.03	0.21
Chol (Pr)	0.28	0.27	0.45*	0.09	0.30	0.45#	0.38*
Chol (St)	0.16	0.17	0.18	0.03	0.15	0.25*	0.21
Chol (Sr)	0.21	0.21	0.28*	-0.01	0.16	0.30*	0.27*
Chol (Sm)	0.10	0.10	0.07	-0.16	0.20	0.27*	0.22
Trig (St)	-0.21	-0.23*	-0.13	-0.19	-0.01	0.07	-0.24*
Trig (Sr)	-0.19	-0.21	-0.13	-0.20	-0.03	0.01	-0.19
HDL (St)	0.16	0.19	0.07	0.14	0.09	-0.03	0.13
HDL (Sr)	0.16	0.07	-0.12	-0.04	0.03	-0.05	0.07
Apo B	0.17	0.15	0.19	-0.06	0.17	0.33*	0.27*
Apo A1	0.20	0.12	0.09	0.05	0.10	0.09	0.21

*p<0.05, #p<0.01, **p<0.001

See Tables 5.1 and 5.3 for abbreviations.

Table 5.5. Correlations between lipoproteins, functional class for angina, age and number of infarcts and normal segments, LV function and collateral supply.

	Norm	LVS	CS	Coll
DOB (years)	0.23*	-0.11	-0.10	0.08
NYHA FC	-0.15	0.05	0.39#	0.33#
No AMI	-0.18	0.42#	-0.03	0.13
Chol (Pr)	-0.46*	0.09	0.36*	0.13
Chol (St)	-0.33#	-0.05	0.09	0.05
Chol (Sr)	-0.38#	-0.03	0.11	0.08
Chol (Sm)	-0.30*	0.04	0.03	-0.09
Trig (St)	0.25*	0.01	0.05	0.02
Trig (Sr)	0.29*	0.11	-0.08	-0.06
HDL (St)	-0.09	0.10	0.02	0.04
HDL (Sr)	-0.01	0.10	-0.12	-0.11
Apo B	0.39#	-0.06	0.07	0.02
Apo A1	0.24	0.05	0.02	0.06

*p<0.05, #p<0.01, **p<0.001

See Tables 5.1 and 5.3 for abbreviations.

Table 5.6. Multiple regression entering apoprotein A1, apoprotein B, the study cholesterol level, triglyceride level, HDL cholesterol level and age as independent variables and the coronary artery scores as the dependent variables.

Dependent Variable	Independent Variable	B	SE B	Beta	Rsq	Sig
NORMSEG	Apo B	-0.0854	0.0260	-0.4469	0.1547	0.0022
CAS	Apo B	0.2220	0.0940	0.3497	0.1224	0.0231
mCAS	Apo B	0.1808	0.0756	0.3535	0.1250	0.0216
mCSS	DOB	-0.9300	0.4535	-0.3083	0.0951	0.0469
JENKINS	DOB	-0.4264	0.2070	-0.3097	0.0959	0.0459
LVS	DOB	-0.1461	0.0575	-0.3726	0.1388	0.0151

B=regression coefficient, Rsq=coefficient of determination.

SE B=standard error of B, DOB=year of birth. See Tables 5.1 and 5.3 for other abbreviations.

Table 5.7. Main Dietary Constituents Measured in Portions.

	CONTROLS		CASES	Diff	95% CI	p	
	Mean	SD	Mean				SD
DAIRY PRODUCTS							
Milk - full	2.9	0.5	1.7	2.5	1.1	-2.4 to 0.01	0.07
- low	2.4	3.9	3.5	5.1	1.1	-0.9 to 3.2	0.28
Addedmilk-high	8.2	12.7	4.0	7.5	4.3	-8.6 to 0.1	0.05*
Addedmilk-low	4.1	9.1	7.3	11.7	3.2	-1.6 to 7.9	0.19
Addedsugar	8.3	12.9	9.3	12.7	1.0	-4.6 to 6.7	0.71
Cream	0.3	0.6	0.2	0.6	0.1	-0.4 to 0.2	0.53
Yoghurt -full	0.4	1.3	0.4	1.1	0.0	-0.6 to 0.5	0.92
-low	0.4	1.2	0.2	0.9	0.1	-0.6 to 0.3	0.53
Cheese -full	3.1	4.0	2.0	3.4	1.0	-2.6 to 0.6	0.20
-low	0.3	1.1	0.4	1.1	0.01	-0.4 to 0.6	0.76
Icecream -full	1.1	1.4	1.0	1.9	0.01	-0.8 to 0.7	0.81
Butter	3.9	8.7	2.5	6.8	1.4	-4.8 to 2.0	0.41
Margarine	20.4	15.2	19.7	11.6	0.6	-6.4 to 5.2	0.83
Eggs - total	1.2	1.7	1.0	1.8	0.2	-1.0 to 0.6	0.63
MEAT							
Total Meat	11.3	5.5	9.4	4.1	1.9	-4.0 to 0.2	0.07
Fried Meat	1.7	2.7	1.6	1.8	0.1	-1.1 to 0.9	0.86
Beef	5.1	3.5	4.0	2.7	1.1	-0.2 to 2.5	0.10
Chicken	2.4	2.4	2.1	1.9	0.2	-1.2 to 0.7	0.65
FISH							
Total Fish	3.0	1.6	2.7	2.6	0.3	-1.5 to 0.9	0.60
GBC Fish	1.4	1.6	1.9	2.2	0.5	-0.4 to 1.4	0.26
Shell Fish	0.4	0.9	0.3	0.7	0.01	-0.4 to 0.3	0.61

GBC=grilled+baked+casseroled, high=full cream, low=reduced fat

Table 5.8. Main Dietary Constituents.

	CONTROLS		CASES		Diff	95% CI	p
	Mean	SD	Mean	SD			
OTHER FAT SOURCES							
Fry/Takeaways	2.1/2.0		1.8/1.8		0.2	-1.1 to 0.6	0.57
Sweets & Bisc	12.5/10.8		9.9/10.1		2.6	-7.2 to 2.0	0.26
Sweets alone	6.6/5.7		3.5/3.3		3.1	-5.0 to-1.1	0.002
Sweet snacks	1.9/3.4		2.3/5.3		0.4	-1.7 to 2.4	0.73
Added oils	1.6/1.9		1.8/3.3		0.3	-1.0 to 1.5	0.68
Nuts	0.6/1.3		0.5/0.9		0.1	-0.6 to 0.4	0.66
SANDWICHES							
Meat	1.9/2.8		1.9/2.4		0.1	-1.1 to 1.2	0.90
Cheese	1.5/2.4		1.1/2.2		0.4	-1.4 to 0.6	0.48
Salad	2.0/3.7		1.6/3.0		0.4	-1.9 to 1.0	0.57
CEREALS							
Total	37.6/17.2		34.8/10.7		2.9	-8.9 to 3.2	0.35
FRUIT							
Total	7.9/7.0		11.6/8.4		3.6	0.2 to 7.1	0.04*
VEGETABLES							
Total	17.2/9.2		18.7/8.7		1.5	-2.4 to 5.4	0.46
SALADS							
Total	2.1/1.8		2.7/2.9		0.6	-0.5 to 1.7	0.30
CAFFEINE							
Coffee	11.9/9.5		10.0/10.01		2.0	-6.3 to 2.4	0.37
Tea	11.6/9.4		11.6/10.0		0.0	-4.3 to 4.3	0.99

Table 5.9. Differences in the dietary constituents (Significant and borderline significant).

	CONTROLS	CASES			
	Mean/SD	Mean/SD	Diff	95% CI	p
TOTAL MEAT	11.3/5.5	9.4/4.1	1.9	-4.0 to 0.2	0.07
SWEETS ALONE	6.6/5.7	3.5/3.3	3.1	-5.0 to -1.1	0.002*
MILK - full	2.9/0.5	1.7/2.5	1.1	-2.4 to 0.0	0.07
- low	2.4/3.9	3.5/5.1	1.1	-0.9 to 3.2	0.28
- added full	8.2/12.7	4.0/7.5	4.3	-8.6 to 0.1	0.05
- added low	4.1/9.1	7.3/11.7	3.2	-1.6 to 7.9	0.19
FRUIT	7.9/7.0	11.6/8.4	3.6	0.2 to 7.1	0.04*

Table 5.10. Previous and current alcohol intake measured in standard portions.

	Controls	Cases	95% CI
Alcohol portions/week (previous estimated)	14.0+/-15.1 (n=44)	18.3+/-20.2 (n=44)	-2.8 to 11.4 p=0.23
Alcohol portions/week (dietary diary)	18.5+/-13.4 (n=32)	14.7+/-13.1 (n=35)	-10.3 to 2.6 p=0.24
Alcohol 24 hrs before	3.2+/- 1.4 (n=17)	3.1+/- 1.4 (n=18)	-0.8 to 1.2 p=0.71

CI=confidence interval of the mean difference

Table 5.11. Previous and current alcohol as stated by the subjects in the two groups.

	Controls	Cases		
Previous Alcohol use	44 (92%)	44 (83%)	p=0.130	OR=3.0
Current Alcohol use	44 (92%)	35 (66%)	p=0.001	OR=7.5
Stopped at diagnosis	-	8 (18%)		
Stopped before diagnosis	-	1 (2%)		

OR=odds ratio

Table 5.12. Difference between the number of current and previous number of subjects using alcohol in the case group.

	Alcohol	No Alcohol	
Previous	44 (83%)	9 (17%)	Chi square=3.18
Current	35 (66%)	18 (34%)	p=0.07
Odds Ratio=2.51 (Yates correction applied)			

Table 5.13. Previous and current alcohol intake as documented in the seven day dietary diary by the subjects in the two groups.

	Controls	Cases	
Previous	44 (92%)	44 (83%)	Chi square=0.01
Diary	32 (66%)	35 (66%)	OR=1.1 p=0.9

Table 5.14. Correlations between the weekly alcohol intake, assessed in standard portions, and CHD risk factors.

	Weekly Intake		Daily Intake	
	Controls	Cases	Controls	Cases
Cholesterol	0.34 #	-0.02	0.42 ##	0.20
Triglyceride	0.30 *	0.34 #	0.34 *	0.14
Apoprotein B	0.21	-0.10	0.29	0.25
LDL cholesterol	0.28 *	0.05	0.40 **	0.26
HDL cholesterol	0.04	-0.21	0.01	-0.09
Apoprotein A1	0.02	-0.10	-0.02	0.11
MCV	0.30 *	0.21	0.27 *	0.10
Packyear	0.38	0.01	0.51 #	0.22
Cigarettes/day	0.32	0.21	0.47 *	0.25
Urate	0.41 **	0.13	0.45 ##	0.12
MPV	-0.20	-0.10	-0.28 *	-0.10
PCR	0.26	-0.01	0.35 *	0.05

* p < 0.05, # p < 0.02, ** p < 0.01, ###p < 0.005

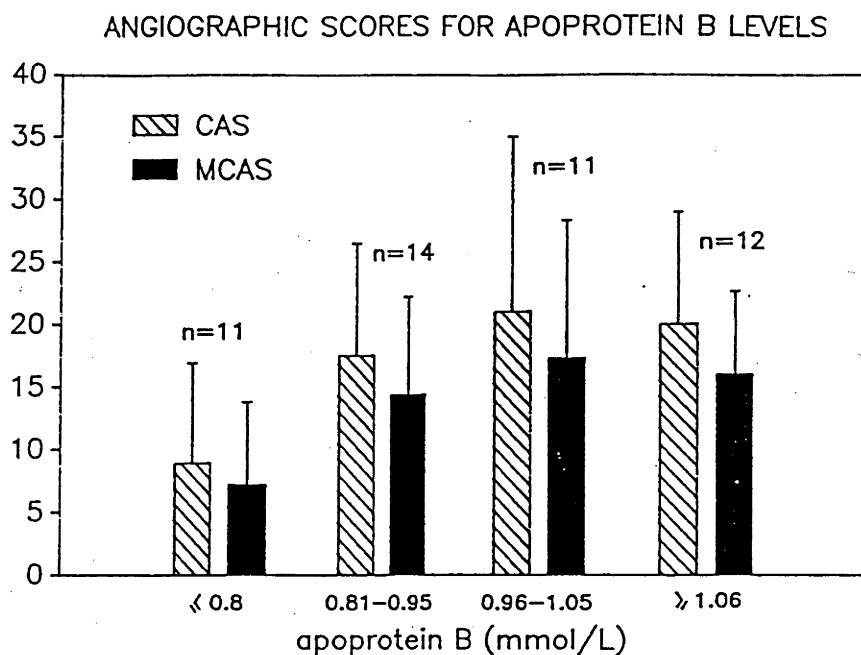


Figure 5.1. Mean coronary atheromatosis score (CAS) and the mean of the averaged coronary atheromatosis score (mCAS) for the subjects when grouped into 4 incremental categories of apoprotein B levels.

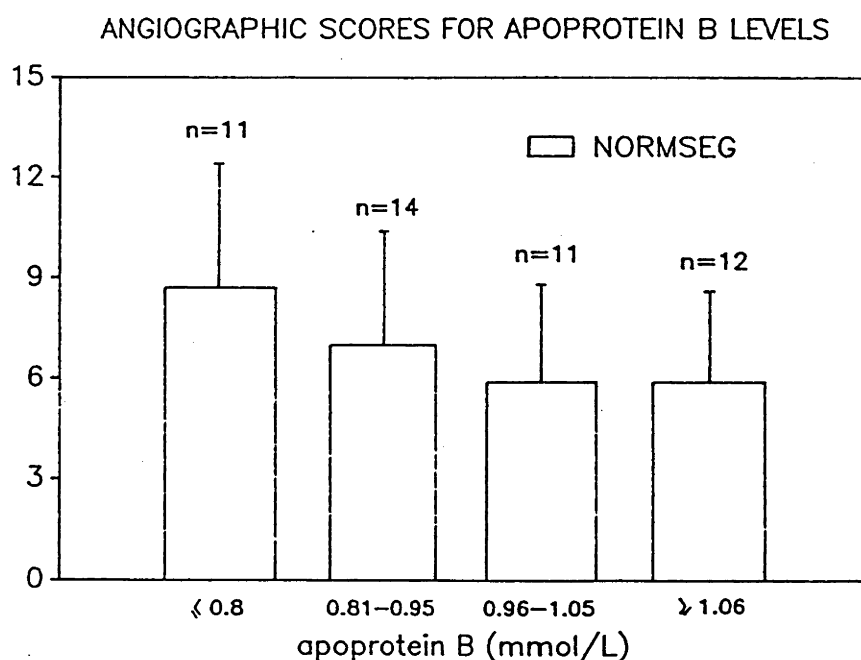


Figure 5.2. Mean number of normal segments (Normseg), coronary artery segments without angiographically visible CAD, for the subjects when grouped into 4 incremental categories of apoprotein B levels.

CHAPTER 6

PLASMA FIBRINOGEN AND CORONARY HEART DISEASE

6.1 THE RELATIONSHIP BETWEEN BLOOD FIBRINOGEN LEVEL AND ANGIOGRAPHICALLY DEFINED CORONARY DISEASE

Blood fibrinogen may have a major influence on platelet function and have a confounding effect on the results of this study. Therefore, an examination of the relationship between fibrinogen level, platelet function measures and CAD was undertaken.

6.1.1 Introduction

An association between CHD and blood fibrinogen level has been found in a number of larger epidemiological reports.⁷⁰¹ These include prospective cohort studies,^{373,702} cohort studies in different populations,^{372,703} population based cross-sectional studies,^{704,705} and case-control studies.^{706,707} Furthermore, persistently elevated fibrinogen levels have been observed in individuals with recurrent MI⁷⁰⁸ and peripheral vascular disease.⁷⁰⁹

Despite these consistent epidemiological reports, the results of studies examining the relationship between the severity of angiographically defined CAD and fibrinogen level have not been uniform.^{374,710-713} A correlation between fibrinogen levels and the number of arteries having haemodynamically significant obstructive CAD lesions³⁷⁴ is controversial.^{711,713} A relationship between mean fibrinogen levels and multi-vessel disease has been observed.⁷¹² In addition, some cumulative scoring systems of CAD severity show an association with fibrinogen levels.^{374,711} while others do not.⁷¹³ These inconsistencies may have resulted from the numerous avenues for bias to give a non-causal relationship, particularly since fibrinogen increases as a non-specific response to a diverse variety of stimuli, including inflammation and tissue injury which are characteristics of atherosclerotic disease.²⁴² Indeed, it is conceivable that raised fibrinogen levels result from extensive atherosclerotic involvement of the arterial system, particularly if there are recurrent cycles of plaque fissuring, fibrin plug formation and repair of endothelial integrity.

This section evaluates the relationship between blood fibrinogen, CHD risk factors and both the extent and the severity of angiographically defined CAD. It is proposed that if the relationship between CHD and fibrinogen is due to a non-specific response to atherosclerosis, those individuals with more extensive CAD will have higher fibrinogen levels compared to those with predominantly localised severe disease without extensive mural involvement.

6.1.2 Methods

6.1.2.1 Patient Population. The patient population studied consisted of the group of 91 male Caucasians, all with documented CAD, undergoing elective coronary angiography for evaluation of CHD. The patient group were selected as a serial angiographic study group. The selection was based on the patient having CHD and documented angiographic CAD. This group was evaluated prior to the case-control study and was investigated to determine the significance of certain less established risk factors such as blood fibrinogen, which is being discussed in this Chapter. The group comprises a different cohort of patients with similar selection criteria to the

case-control study except there was no age limitation. The mean age was 53.2 years (range 35 to 69 years). Patients were excluded on the following criteria: myocardial infarct within 5 weeks, valvular heart disease, right heart failure or current use of anticoagulants; recent infection, surgery or trauma; any inflammatory disease, diabetes, overt renal disease, liver disease or coagulopathy. The patients were unselected with respect to the severity, nature or duration of symptoms and concurrent drug therapy, apart from anticoagulant use.

6.1.2.2 Angiographic Scoring Methods. Coronary angiograms were graded to give a measure of the severity and extent of atherosclerotic disease by a semi-quantitative method.²⁰⁷ The methodology is described in detail in Chapter 8.

6.1.2.3 Laboratory Measurements. Laboratory methods used are described in Chapter 2.

6.1.2.4 Statistical Analysis. All statistical methods used are described in Chapter 2. The association between the fibrinogen level and CAD after adjustment for other major risk factors was further assessed using analysis of covariance. For the analysis of covariance the fibrinogen was defined as the continuous dependent variable, the coronary artery scores the independent factor categorised into 4 groups, and the major risk factors as the covariates. The time since the last MI, the number of infarcts reported and the time from cessation of smoking were skewed to the right and were therefore logarithmically transformed before parametric analysis. The fibrinogen distribution conformed closely to a normal distribution in our population (mean=314, median=300 and skewness=1.105).

6.1.3 Results

6.1.3.1 Patient Characteristics. The group values for the major risk factors and the results of the angiographic scores are summarised in Table 6.1. Only 2 patients were currently smoking and 81% had formerly been tobacco users. The mean interval since cessation of tobacco use was 7.3 (SD+/-12.1) years, although 44% had only ceased within one year and 65% within 5 years. The mean cholesterol level was 5.62 (SD+/-0.96) mmol/l. Twenty seven percent of patients had a triglyceride level over 2.0 mmol/l and 36% with a HDL cholesterol less than 0.9 mmol/l. Only 19% of the patients had been informed of a diagnosis of hypertension. One had a systolic BP greater than 140 mmHg and three patients had a diastolic pressure greater than 90 mmHg whilst on drug therapy. All patients were on some form of medication; 45% were using beta-blockers, 46% were on calcium entry blockers, 30% were on nitrates, 8% were on diuretics, 2% were on digoxin and 1% on antiarrhythmics. There were no subjects with a fibrinogen level below the reference range for the laboratory (150mg/ml), although 19 patients were above this range (400mg/ml).

Seventeen patients gave no history of a previous acute AMI, 55 patients had one AMI, 12 claimed to have had 2 AMIs and 4 patients had had 3 AMIs. Three patients were uncertain. The ECGs of 58 patients were consistent with a previous AMI and 28 were indeterminate. Abnormalities on the left ventriculogram indicative of a previous MI were seen in 68 patients. The mean time from an AMI was 240 days (range 35 to 2990 days, median 110 days, earliest 35 days). Fourteen patients de-

scribed no angina, 31 had a variable level of angina, 30 had a predictable level of exertional angina with normal activity, 13 had angina on minimal exertion and 3 described intermittent rest pain.

6.1.3.2 Fibrinogen and CHD Risk Factors. For descriptive purposes, fibrinogen levels were divided into four groups of increasing amount with approximately equal numbers for a graphic examination between fibrinogen and other CHD risk factors (Fig. 6.1 and Fig 6.2). Possible associations between fibrinogen and the risk factors were examined by linear correlation (Table 6.2). The amount of previous chronic cigarette use, the triglyceride level and age were directly related to the fibrinogen level in patients with CHD (Table 6.2). The relationship between fibrinogen and current tobacco use could not be evaluated since only 2 patients admitted to continuing smoking of tobacco. The fibrinogen level was also inversely and independently related to the HDL cholesterol level (Table 6.2). There was no relationship between body weight and fibrinogen level.

Multiple regression analysis using fibrinogen as the dependent variable demonstrated an independent association between the fibrinogen level and three major risk factors for CHD; previous cigarette smoking and age were directly, and the HDL cholesterol level inversely associated with the fibrinogen level (Table 6.3). Hypertension, cholesterol and triglyceride levels did not demonstrate an independent predictive association for fibrinogen level with multiple regression analysis.

6.1.3.3 Age, Risk Factors and Angiographic Score. The age of the patient correlated with the level of cholesterol, the systolic BP and the fibrinogen level, and inversely with the HDL cholesterol level (Table 6.2). There was a clear predictable relationship between age and many of the angiographic scores (Table 6.2). In particular, a direct but greater correlation between the extent of CAD (CAS) than the severity (CSS), a direct correlation with the number of segments with diffuse disease and with calcification, and an inverse relation to the number of normal segments were present (Table 6.2). These associations are consistent with known and expected relationships between age, CAD and risk factors, and give an indication as to the validity of the methodology used.

An independent predictive association between the other major risk factors and the CAD scores by multiple regression analysis was not demonstrated in this study. The only risk factor predictive of the CAS and CSS in the multiple regression analysis was age ($B=6.8859$, $SE\ B=3.2955$, 95% confidence interval 0.2667 to 13.5051, $p=0.04$; and $B=14.7407$, $SE\ B=5.7151$, 95% confidence interval 3.2616 to 26.2199, $p=0.01$ respectively for CAS and CSS).

6.1.3.4 Fibrinogen and Coronary Artery Disease. An illustrative presentation of the relationship between fibrinogen, divided into four subgroups, and the measures for extent (CAS) and severity (CSS) of CAD is depicted in Fig 6.3. There is a clear increase in the severity score with increasing fibrinogen level when comparing the lower and higher groups. There was a modest but significant direct univariate correlation between fibrinogen and the number of stenosis $>50\%$ and $>70\%$, the number of coronary artery occlusions, the CSS and the collateral score (Table 6.2). This association between fibrinogen and severity was not present when fibrinogen

was entered into a multiple regression model after adjusting for other risk factors for CHD (viz, age, cigarette packyears, cholesterol, HDL cholesterol and triglyceride levels). In this model, the dependent variable was the severity measure and the independent variables were fibrinogen and the other risk factors.

The converse was found when fibrinogen was considered the dependent variable. The level of fibrinogen was found to be partially dependent on, and predicted by the severity of CAD (CSS) along with the age of the patient, volume of previous cigarette use, and the inverse of the HDL cholesterol level, but not the extent of disease (CAS) or the severity of left ventricular dysfunction (LVS) (Table 6.3). These significant associations by multiple regression analysis remained significant for those patients not taking beta-blockers or calcium entry blockers.

6.1.3.5 Fibrinogen, Myocardial Infarction and Left Ventricular Damage.

Fibrinogen levels had a modest direct association with the number of AMIs and also with the number of total arterial occlusions demonstrated angiographically, and the score of wall motion abnormalities (LVS) (Table 6.2). Those patients who had evidence of left ventricular damage as measured by wall motion abnormalities on the ventriculogram had a higher level of fibrinogen than those with normal function (Table 6.4). The group with the highest levels of fibrinogen had on average a significantly greater degree of LV dysfunction as supported by a direct univariate correlation between fibrinogen and LVS (Table 6.2). Those with LV damage also had a greater volume of cigarette intake during their life (Table 6.4). None of the other major risk factors assessed were related to the LVS. Furthermore, there was no relation between fibrinogen level and the time since the last AMI.

Multiple regression analysis failed to confirm an independent association between LV damage and fibrinogen levels when the severity score and other risk factor variables were taken into account. The only independent variable associated with the LVS determined by multiple regression analysis was the measure of severity of CAD (CSS).

6.1.4 Discussion

In males with established CHD, a modest direct linear association exists between fibrinogen levels and the severity of CAD but not the extent of coronary atheroma. The fibrinogen level in this group of CAD patients is partly dependent on other risk factors (age, smoking and HDL cholesterol level) as well as the presence of severe stenoses. Given this last observation, it is feasible that an underlying disease process may concomitantly cause severe intraluminal obstructive lesions and increased fibrinogen levels, such as recurrent mural thrombosis and fibrin formation within and/or over atherosclerotic plaques.^{639,714}

A descriptive assessment of the relationship between fibrinogen and other variables was made by dividing fibrinogen into 4 groups with an increasing range of values. This descriptive illustration supports the modest linear relationships shown in the correlation assessment, and, more importantly, demonstrates the poor clinical utility of the fibrinogen level for predicting severity or extent of disease in an individual. Other studies have also shown a marked overlap of the fibrinogen levels between

groups having increasing CAD severity.³⁷⁴ The fibrinogen level in such groups also had a large standard error of the mean.³⁷⁴

Previous studies evaluating the relationship between fibrinogen and angiographically defined CAD have made conflicting observations. A significant association between fibrinogen and a measure of CAD has been documented previously⁷¹¹ and multivessel CAD disease patients have higher fibrinogen levels than patients with single vessel disease.⁷¹² Another study, using multiple linear regression, failed to show any predictive relationship between fibrinogen and angiographic CAD.⁷¹³ The disparities between these reports, and with the current study, most likely arise from methodological differences, particularly in relation to the method of defining CAD. For example, the scoring system criteria used in one study⁷¹³ was derived from clinical requirements and lesion haemodynamics, and does not provide a broad gradient measure of overall extent and severity of atherosclerosis. In a more recent study,³⁷⁴ which reported plasma fibrinogen as being an independent predictor of CAD severity, a number of methodological anomalies exist. For example, alcohol intake was entered into the multivariate analysis used in the study even though it was not correlated with fibrinogen levels and it is an equivocal risk factor for CAD. Yet triglyceride levels, which may influence the relationship,⁷¹³ were not entered. Furthermore, patients with severe left main stem arterial lesions were excluded, ex-smokers of 5 years or more were considered non-smokers, controlled diabetics were included, and, even though the blood sugar univariately correlated with fibrinogen, it was not entered into the multiple regression analysis. Also, approximately 33% of the male group studied did not have significant obstructive CAD (Gensini score of zero). Despite these patient population and methodological differences, both our observations and this report³⁷⁴ support the presence of an independent association between severe disease and fibrinogen level, with fibrinogen as the dependent variable. Unfortunately neither study can clarify whether the association provides an aetiological link between plasma fibrinogen and obstructive CAD.

The degree of functionally impaired myocardium has a direct and proportional association with fibrinogen. A negative correlation has previously been demonstrated between fibrinogen and left ventricular ejection fraction.⁷¹³ This observation is in agreement with the positive association between the LVS and fibrinogen level. However, this association would appear to be indirect rather than causal, since the only variable independently associated with our measure of left ventricular dysfunction (LVS) by multivariate analysis is disease severity, the fibrinogen level only being related by association with disease severity (CSS).

An increase in fibrinogen occurs in the acute phase of a MI.⁷¹⁵⁻⁷¹⁷ In general fibrinogen reaches a maximum level within the first week^{715,716} and in most cases returns to pre-infarct levels within 3 to 4 weeks.⁷¹⁵ This time course parallels that for the healing of necrotic tissue within the myocardium following an infarct.¹¹ This study has demonstrated an association between fibrinogen and left ventricular segmental dysfunction present beyond such a time frame. However, our multiple regression analysis would suggest that chronically elevated levels are more directly related to the underlying disease severity. Similarly, the univariate relationship of fibrinogen with the number of AMI probably arises indirectly via disease severity, or by mechanisms simultaneously leading to severe lesion development, AMI, and in-

creased fibrinogen production.

Previous studies have established an association between fibrinogen and a number of the major risk factors for CHD. These factors include age,^{705,718-720} tobacco use,^{327,703,718-721} blood cholesterol level,^{372,631,721} and lower socioeconomic status.⁷²² We have confirmed the presence of an independent direct relationship between the fibrinogen level and previous cigarette use by an individual. This study cannot evaluate the acute effects of cigarette smoking on fibrinogen levels. The association was with the total volume of cigarette intake during the life of the patient, supporting the observation that it may take more than 5 years for fibrinogen to return to pre-smoking levels.⁶⁰ It is of relevance that ex-smokers may be at higher risk of acute coronary events for at least 15 years after stopping.⁷²³ Furthermore, it has been suggested that a substantial part of the relationship between smoking and CHD may be mediated through fibrinogen.⁶⁰ Equally, the converse may be true. Our findings do not resolve this question but do demonstrate that long-term smoking directly results in a dose dependent increase in fibrinogen. However, the apparent relationship between the chronic use of cigarettes and fibrinogen level may be the result of observer/selection bias. There was no validation of the accuracy of the self-reporting of tobacco intake, including the current use of cigarettes. Therefore, the association may feasibly be related to acute smoking effects that were unreported.

An unexpected observation was the relatively strong and independent inverse association with HDL cholesterol levels. Most previous reports concerning individuals with or without CHD have not examined such a possible relationship, or possibly did not report the lack of association,^{705,711-713} and little is available from population based studies.^{372,373,703,718,722} A lack of association has been reported for a normal population group,⁷¹⁹ and also for CHD patients.^{374,704} A negative but insignificant association using the 'clottable' fibrinogen method but not with the heat precipitation method for fibrinogen measurement has been demonstrated in CHD patients randomly selected from a specific population.⁷⁰⁴ The relationship must remain a tentative possibility but does highlight the importance of validation techniques for self reporting of tobacco intake to assess under-reporting of past and current tobacco use. This is of further importance with regard to the HDL cholesterol level association with fibrinogen. Smoking has a well known inverse association with HDL cholesterol levels, and under-reporting may introduce sufficient bias to give a false independent association between fibrinogen and HDL cholesterol, and reduce the possibility of measuring an association between smoking and the severity of CAD. The inverse association observed in our study indicates an area for further study. Nevertheless, the observation has subsequently been confirmed in other studies.⁷²⁴⁻⁷²⁹, although the reason has not been elucidated. The later uniform confirmation of the positive association between HDL cholesterol and fibrinogen level adds further validation to the observations of this study.

Increasing age is one of the most powerful predictors for the development of both CAD and CHD.²⁰² This is reflected in the results of our univariate and multivariate analysis. Population based fibrinogen levels have a positive relationship with age,⁷³⁰ which also occurs in aging CHD patients.⁷³¹ It is possible that any increase in fibrinogen with age noted in population based studies may in part be due to the development of subclinical severe obstructive disease. This would increase the likelihood of

the fibrinogen level being a reliable prospective predictor of CHD and symptomatic events in a "normal" population group.

Apart from age, the risk factors analysed in the present study do not significantly contribute to the variance of disease severity or extent within a group of males with established CHD. This observation does not negate their possible influence as risk factors for the initiation of CHD in a susceptible individual or group, and this applies equally to the fibrinogen level. However, within such a group they do not appear to contribute substantially to the severity and extent of disease expression as interpreted angiographically. If the study sample number were to be increased greatly in order to detect possible weak associations which were significant in the multiple linear regression analysis, their contribution to the variance of the dependent variable would not be remarkably altered. Therefore, even if the sample size contributed to a type II statistical error, increasing the number would not result in a relationship that had a substantial clinical value.

A potentially major confounding methodological problem with most angiographic studies, including the presently reported observations, is the lack of incident cases used to assess the relationship. Lipid measures, hypertension, weight and tobacco use will all have undergone possible modification following the initial diagnosis, leading to a selection bias and hence limiting the likelihood of necessarily finding significant associations. In addition, modification of both the underlying disease as well as the risk factors may occur following the long-term use of cardiovascular active drugs, such as beta-blockers and calcium entry blockers. This potential problem may also apply to this assessment of fibrinogen despite the exclusion of individuals with known confounding factors. A possible important association between diet and fibrinogen may exist. A direct relation between linoleic acid intake⁶⁶⁰ and an inverse association with dietary cereal⁷¹⁹ have been reported. No major influence over fibrinogen levels has been noted for cardiovascular medications.^{60,418} Those drugs recorded as being able to modify fibrinogen levels⁷³² were not used by the patients with CAD evaluated in this study. A prospective angiographic study is required to more appropriately evaluate the observations from our cross-sectional study, but with current angiographic methodology a prospective study on normals to more reliably evaluate a causal relationship cannot presently be performed.

6.1.5 Summary

1. The above observations do not verify the proposition that an increased fibrinogen level is causally associated with a greater extent and/or increased severity of CAD. Elevated fibrinogen levels do have a modest relationship with severe CAD. This relationship, however, may be due to another process simultaneously leading to increased fibrinogen levels and the development of severe stenotic CAD, such as increased platelet reactivity. The plasma fibrinogen level must be taken into account when evaluating differences in platelet function between any two groups.

2. They indicate that any relationship existing between fibrinogen and CAD is unlikely to be due to a non-specific response to atherosclerotic involvement of the coronary arteries. An independent association between fibrinogen and the extent of coronary arterial wall involvement, and by inference, atherogenesis, was

not able to be demonstrated.

3. The fibrinogen level for an individual with CAD is in part directly predicted by the volume of previous cigarette use, the age of the individual, the severity of underlying CAD, and inversely by HDL cholesterol level.

4. The results remain consistent with the observation that *a priori* high fibrinogen levels may be predictive of an increased incidence of CHD events. This predictive relationship would appear to be mediated through an association with other risk factors and with CAD severity. Whether there is any causal relationship between CHD events and fibrinogen levels still remains to be determined.

6.2 FIBRINOGEN LEVELS IN YOUNG MALES WITH CORONARY HEART DISEASE

6.2.1 Introduction

Unequivocal epidemiological evidence exists confirming an elevated blood fibrinogen level as a risk factor for CHD.^{372,373,701-707} However, as indicated in section 6.1, whether or not an elevated fibrinogen level is a causal risk factor has not been firmly established. Raised fibrinogen levels may result from recurrent plaque fissuring which occurs in subjects with coronary atheroma.⁷³³ This plaque fissuring can lead to the episodic growth of coronary stenoses, the plaque expansion initially being due to thrombosis within the lesion.⁷³³ In addition, there is a substantial body of evidence supporting the early involvement of coagulation products, fibrinogen and fibrin in particular, in the formation of atheroma.^{539,714,734-736}

One of the epidemiological criteria required to establish causality is to show a biological gradient between the factor and the outcome.^{470,737} A univariate association between the fibrinogen level and the severity of angiographically defined CAD⁶¹ as described in section 6.1 of this thesis supports this concept. The relationship, however, was not independent of age, previous cigarette use and other risk factors for CHD using multivariate analysis.⁶¹ In the past, similar methods of assessing the relationship have been used by other groups, although the results of those studies examining the relationship between the severity of angiographically defined CAD and fibrinogen level have not been uniform.^{374,710-713} There are numerous potential areas of bias to account for these inconsistent results, particularly since fibrinogen increases as a non-specific response to a diverse variety of stimuli,⁷³⁸⁻⁷⁴⁰ including inflammation and tissue injury characteristic of atherosclerotic plaques.²⁴² Nevertheless, it remains uncertain whether the interaction of fibrinogen with ruptured plaques results in the increased plasma level, whether CHD risk factors account for all the elevation, or whether a preexisting increased level potentiates thrombus formation within the plaque or arterial lumen.

Another criteria for inferring causality is that the disease should follow exposure to an increased level of the postulated causative factor, and an increase in magnitude of the disease after the exposure.⁷³⁷ If an increased level of fibrinogen is associated with a greater likelihood of plaque expansion due to thrombosis within the lesion,

then an elevated fibrinogen level may predate any association of fibrinogen with CAD severity in the early stages of CAD. Therefore, in this section, the relationship between fibrinogen levels and angiographically defined CAD in non-diabetic young males with premature CHD is evaluated.

6.2.2 Methods

6.2.2.1 Patient Population. The case and control groups consisted of the young males who were age, race and socioeconomically matched as described in Chapter 3. The exclusion criteria are also described in Chapter 3 and section 6.1 of this thesis.

6.2.2.2 Laboratory measurements. The laboratory methods have been described previously⁶¹ and are detailed in Chapter 2.

6.2.2.3 Angiographic Scoring Methods. Coronary angiograms were graded to give a semi-quantitative measure of severity and extent of angiographically defined CAD, as previously described^{61,207} and detailed in Chapter 8.

6.2.2.4 Statistical Analysis. Statistical methods used are described in Chapter 2.

6.2.3 Results

6.2.3.1 Group comparisons. The mean values for the major risk factors are summarised in Table 3.1. All tobacco users were cigarette smokers and there were 7 and 4 current smokers in the control and case groups respectively. There was a significant and predictable difference between the groups for all the major CHD risk factors. The fibrinogen was also significantly greater in the case group (275 \pm 10 vs 230 \pm 8 mg/100ml, $p=0.001$).

6.2.3.2 Correlations. The fibrinogen level in both groups correlated weakly with age, and the age distribution of both groups was narrow (Table 6.5). In the case group, the fibrinogen level correlated directly with body weight, BMI, scapular skin fold thickness, blood glucose level, HbA1C and Hb levels, and inversely with HDL cholesterol levels. The control group fibrinogen level correlated directly with Hb, Hc and apoprotein B levels (Table 6.5).

6.2.3.3 Multivariate analysis. Following multivariate analysis, with fibrinogen as the dependent variable and those factors having a univariate association as independent variables, the Hb remained predictive of fibrinogen levels in the control group, and scapular skin fold thickness remained predictive in the CHD group (Table 6.6).

6.2.3.4 CAD angiographic scores. The fibrinogen level did not correlate with any of the angiographic measures of CAD in the multivariate analysis.

6.2.4 Discussion

The results of this study demonstrated that an increased plasma fibrinogen level in males with CHD predates any measurable association with CAD severity, an associa-

tion demonstrable in older male patients with established CHD.⁶¹ These results, in conjunction with the previous observations (section 6.1),⁶¹ were consistent with the concept that increased fibrinogen levels are involved in the development of severe lesions which form over time in males prone to CHD. Since the increased level was present before an association was demonstrable, another epidemiological criteria for causation is satisfied. That is, an increase in the magnitude of the disease should follow exposure to an increased level of the postulated causative factor.^{470,737}

However, even though the observations support the above concept, the results are from two different groups and from separate cross-sectional studies. Therefore, other explanations are clearly possible. For example, it is feasible that the disease process may concomitantly cause severe intraluminal obstructive lesions and increased fibrinogen levels, due to recurrent intramural and extramural thrombosis and fibrin formation.^{61,639,714} In addition, an association between fibrinogen levels and CHD risk factors is also present at this earlier stage of disease development. Previous studies have established an association between fibrinogen and a number of the major risk factors for CHD including age,^{705,718-720} tobacco use,^{372,703,718-721} blood cholesterol,^{372,631,721} obesity,⁷³⁸ diabetics,^{143,738} blood sugar level in non-diabetics,^{143,374} lower socioeconomic status,⁷²² and a number of other factors.⁷³⁸ The fact that not all of these associations were present in the present study group is most likely attributable to the specific population groups studied. Also the size of the study group would only allow the detection of more robust relationships and a type II statistical error probably accounts for the inability to demonstrate some relationships. Those relationships shown would more likely to be of clinical relevance. Nevertheless, an association with risk factors for CHD may still explain the increased fibrinogen occurring in those with premature CHD.

An association between blood glucose and fibrinogen levels in a non-diabetic population has been well documented.¹⁴³ The association between fibrinogen and HbA1C is novel, but not unexpected.¹⁴³ A weak relationship with age exists in both groups, and the narrow age range in both groups probably explains the minor association.

An important feature emerging from this study is that the factors associated with fibrinogen levels differ for the males with premature CHD compared to the matched controls without CHD. Of particular note, there is a cluster of factors (blood glucose level, HbA1C, BMI, body weight and scapular skin fold thickness) related to fibrinogen in the univariate correlation not present in the control group. A difference is also present after multivariate analysis. An independent relationship with a measure of central body obesity, namely scapular skin fold thickness, is unique to the case group. One likely explanation for such an unusual relationship is that other factors not measured (eg genetic variation) influence the fibrinogen level in one group and not the other. The relationship is evaluated in more detail in the following section. This study highlights the importance of having a greater understanding of the mechanisms, including genetic, which control plasma fibrinogen levels, since the factors related to fibrinogen level in two homogeneous and matched groups differ.

8.2.5 Summary

1. Fibrinogen levels are higher in young male patients after the diagnosis

of CHD than in controls without CHD. The elevated fibrinogen is not associated with angiographically defined CAD in the young CHD group.

2. Factors associated with the fibrinogen levels differ between controls and cases, with a cluster of factors, viz blood glucose level, HbA1C, BMI, body weight and scapular skinfold thickness, being associated with fibrinogen levels in the case group.

6.3 POSSIBLE MECHANISMS FOR THE ASSOCIATION BETWEEN PLASMA FIBRINOGEN AND CHD. PLASMA FIBRINOGEN LEVELS, LIPOPROTEINS AND BLOOD GLUCOSE IN YOUNG MALES WITH CORONARY HEART DISEASE

6.3.1 Introduction

It is clear from the results presented in this Chapter, and from the literature, that the epidemiological evidence for an elevated blood fibrinogen level being a risk indicator for CHD is unequivocal. Nevertheless, a mechanism for the relationship has not been elucidated and the question as to whether there is a causal relationship has not been fully established.^{62,372,373,701-707} Moreover, there exists a clustering of the other major risk factors that are associated with plasma fibrinogen level, the mechanisms of which also remain unexplained. An association between fibrinogen and other major risk factors for CHD has been well established.^{62,143,372,374,631,703,705,718-722,738} In addition, an independent inverse association between plasma fibrinogen and HDL cholesterol levels in males with CHD was noted in the preliminary studies for this thesis.⁶¹ At that time most previous reports concerning individuals with or without CHD had not examined the relationship.⁶¹ Indeed, review articles made no mention of a relationship.^{62,738,740} One possible explanation was that a negative association had not been reported.^{705,711-713} There was also little available information from population based studies.^{372,373,703,718,722} There were reports demonstrating no association in a normal population group⁷¹⁹ and in CHD patients.³⁷⁴ A positive association with CHD patients using the 'clottable' fibrinogen measurement method had been demonstrated, but not with the heat precipitation method.⁷⁰⁴ However, since then the association has been substantiated by a number of studies which recently have been reported in preliminary form.⁷²⁴⁻⁷²⁹ In population studies, the fibrinogen level correlated positively with total cholesterol,⁷²⁴ also with LDL cholesterol,^{725,728} and inversely with HDL cholesterol levels.^{724,725,727,728}

Associations between plasma fibrinogen level and obesity⁷³⁸ and diabetes^{143,738} are well recognised, and even with the blood glucose level in non-diabetics.^{374,143} Of additional interest is an apparent association between plasma fibrinogen and blood insulin levels,^{727,728} as well as body fat distribution.^{724,727,741-743} However, not all studies demonstrate an association with fat distribution⁷⁴⁴ or do so only with univariate analysis.⁷²⁷

Therefore, in light of the above information, in this section the relationships of plasma fibrinogen levels in non-diabetic young males with premature CHD and the well matched control group are further evaluated in order to further refine our

understanding of the relationships.

6.3.2 Methods

6.3.2.1 Patient Population. The case and control groups consisted of the young males defined in Chapter 3. The exclusion criteria are described in section 6.1.2.1.

6.2.2.2 Laboratory measurements. The laboratory methods have been described previously⁶¹ in Chapter 2.

6.3.2.3 Anthropomorphic Measures. These measurements are described in full in Appendix 2.

6.3.2.4 Glycosylated Haemoglobin. A colourimetric method was used for the estimation of Hb glycosylation³⁶⁴⁻³⁶⁶ and is described in Chapter 2.

6.3.2.5 Statistical Analysis. Associations were evaluated by univariate analysis and the details are given in Chapter 2.

6.3.3 Results

6.3.3.1 Group comparisons. The mean values for the major risk factors are summarised and comparisons made between the groups in Chapter 3. Of particular note, was that all tobacco users were cigarette smokers and there were 7 and 4 current smokers in the control and case groups respectively. In addition, the non-diabetic case group had a significantly higher BSL (5.3 ± 0.10 vs 4.9 ± 0.08 mmol/l, $p=0.02$) and HbA1C level (7.8 ± 0.2 vs 7.3 ± 0.1 , $p=0.03$) than the controls, although the levels were within the normal range. Moreover, the scapular skin fold thickness was significantly higher in the case group (17.1 ± 0.9 vs 1.8 ± 0.7 cm, $p=0.006$), but there was no difference in the weight, height, BMI and triiceps skin fold thickness. Finally the plasma fibrinogen level was also significantly greater in the case group (275 ± 10 vs 230 ± 8 mg/100ml, $p=0.001$).

6.3.3.2 Correlations. The fibrinogen level in both groups correlated weakly with age but the age distribution of both groups was narrow (Table 6.5). The correlation with other factors is described in section 6.2.

6.3.3.3 Multivariate analysis. Multivariate analysis was performed with the plasma fibrinogen level as the dependent variable. Those CHD risk factors which had a univariate association were entered into the regression equation as the independent variables. The Hb remained predictive of fibrinogen levels in the control group. Scapular skin fold thickness was independently predictive of fibrinogen level in the CHD group (Table 6.7).

6.3.4 Discussion

An independent inverse association between plasma fibrinogen and HDL cholesterol levels has been documented in a broad age group of patients with CHD (Section 6.1).⁶¹ This observation has been supported by the early reports from a number of

other studies.⁷²⁴⁻⁷²⁹ In the present report of a younger group of males with premature CHD, an inverse univariate association was similarly demonstrated, but was not independently predictive of the risk factors measured. More importantly, the association was not demonstrated in the normal controls. However, in the preliminary study described in section 6.1,⁶¹ HbA1C and scapular skin fold thickness were not measured, and hence not included in the multivariate analysis. From the present results, it would appear that the relationship between fibrinogen and HDL cholesterol levels may be due to a co-morbidity bias. That is, both are associated with each other through an association with the cluster of risk factors of central body obesity, abnormal glucose homeostasis, hypertension and abnormal lipoproteins. Indeed, that link could also be the reason that they are risk factors for CHD.

The inverse association between fibrinogen and HDL cholesterol levels can also be supported by a plausible biological explanation, and may well be a direct association. A strong positive relationship between plasma lipoprotein lipase (LPL) and HDL cholesterol levels has previously been documented.⁷⁴⁵ Thrombin inhibits LPL²¹⁰ and also appears to stimulate fibrinogen synthesis by an enzymatic mechanism independent of fibrinogenolysis or disseminated intravascular clotting.⁷⁴⁶ Therefore, increased thrombin activity, for whatever reason, may increase fibrinogen production and increased fibrin breakdown products will further stimulate fibrinogen synthesis. The increased thrombin activity will also inhibit LPL activity possibly leading to decreased HDL cholesterol levels. Furthermore, fatty acids significantly increase the production of fibrinogen,²⁰⁸ while polyunsaturated fatty acids and triglycerides decrease HDL cholesterol levels.⁷⁴⁷

An association between the blood glucose and fibrinogen levels in a non-diabetic population has been documented in the past,¹⁴³ and an association between fibrinogen and HbA1C values had been previously predicted.¹⁴³ There exists a cluster of CHD risk factors (blood glucose level, HbA1C, BMI, body weight and scapular skin fold thickness), which together are measures of a group of risk factors which are well recognised predictors for CHD.^{748,749} However, the fibrinogen level univariately correlated with the factors in this grouping only in the CHD group, and the association was not present in the control group. Furthermore, the strength of this relationship was demonstrated when a measure of central body obesity, namely scapular skin fold thickness, was independently predictive of the fibrinogen level, an observation also unique to the CHD group.

An elevated fibrinogen level in populations with a low incidence for CHD is not a risk factor for CHD.⁶⁰ High fibrinogen levels occur in groups where CHD disease is rare, suggesting that if increased fibrinogen levels cause CHD, high levels must interact with some other characteristic.⁶⁰ Fibrinogen has a strong relationship with hyperglycaemia in diabetics¹⁴³ and clusters, along with other CHD risk factors, with an abnormal lipoprotein profile.⁷⁵⁰ As discussed, the fibrinogen level, in addition, directly correlates with a number of other risk factors. These facts, in combination with the observations made in this report, that the fibrinogen level in CHD patients with premature CHD is related to specific risk factors and is not related to the same factors in a normal group, supports the concept that an elevated fibrinogen level is possibly only a causal risk factor for CHD (that is the clinical expression of the disease) in individuals with developed CAD having preexisting atheroma, as pre-

viously suggested.⁶⁰ Alternatively, the association may be through mechanisms giving abnormal glucose homeostasis, abnormal lipoproteins (including low HDL cholesterol levels), hypertension and central body obesity, which so often cluster together in individuals with CHD.

6.3.5 Summary

1. An inverse association between HDL cholesterol and fibrinogen levels in patients with CHD was demonstrated in preparatory study of this Thesis and confirmed in the main case-control study. This was a novel observation which has also been reported independently by others. The mechanism of this association remains to be elucidated.

2. In CHD patients the fibrinogen level appears to have an association with other more recently recognised risk factors which cluster together, viz central body obesity, abnormal glucose homeostasis and lipid abnormalities. Given these observations, the impact of such potential relationships on the results of this Thesis are examined in Appendix 2.

6.4 FIBRINOGEN AND *IN VITRO* PLATELET AGGREGATION

6.4.1 Introduction

6.4.1.1 **Fibrinogen's role in platelet aggregation.** Fibrinogen is essential for platelet aggregation and occupancy of a receptor by fibrinogen, which occurs only after platelet stimulation, is involved in the primary pathway for platelet aggregation.⁷⁵¹⁻⁷⁵⁴ Fibrinogen binding to human platelets and platelet aggregability are directly related.^{306,754} The platelet membrane GP IIb/IIIa acts as a receptor for fibrinogen⁷⁵⁵ and forms a complex with purified plasma fibrinogen and may act as the fibrinogen binding site required for normal platelet aggregation.⁷⁵⁶ Prostaglandin endoperoxides and thromboxane may play a role in the exposure of fibrinogen receptors on normal platelets.⁷⁵⁷ Bound fibrinogen then mediates platelet aggregation by bridging platelets and/or serving as a cofactor for the aggregating process.⁷⁵⁸ However, the precise mechanism how platelet bound fibrinogen is involved in aggregation is not fully established.⁴¹⁸

Fibrinogen binds to receptors, which are specific and saturable, on the platelet surface during platelet aggregation. The receptors are not exposed on unstimulated platelets and fibrinogen is unable to bind to unstimulated circulating platelets (Reviewed in references^{758,759}).

Fibrinogen is present on the surface of the platelet. It is also present intracellularly, within the alpha-granules from where it is released by an energy requiring secretory mechanism which occurs in response to aggregating agonists such as thrombin, collagen and ADP.⁷⁵⁹ The exact function of intracellular platelet fibrinogen is currently unknown although afibrinogenic platelets appear to function normally with exogenous fibrinogen.⁷⁵⁹

6.4.1.2 Effect of platelet aggregating agents. ADP stimulation results in rapid reversible binding of exogenous fibrinogen to the receptors after platelet stimulation. The effect of fibrinogen is greater at lower concentrations of ADP and reversed at higher levels.^{331,760} Platelet fibrinogen secretion arises after aggregation induced by ADP, which is dependent on the secretory mechanism.

Aggregation induced by thrombin, however, is independent of a secretory response, either preceding or occurring simultaneously with aggregation.⁷⁵⁹ Although fibrinogen binding to stimulated platelets is much greater than that to unstimulated platelets regardless of the agonist used, thrombin stimulation induces binding which is much greater than that induced by ADP or adrenaline (Reviewed elsewhere^{758,759}). The GP IIb/IIIa receptor appears to play an important role in agonist-induced aggregation of platelets.^{755,761}

Even if binding of fibrinogen is a prerequisite for platelet aggregation, additional conditions determine the proaggregating properties of fibrinogen.⁷⁶² Interaction of fibrinogen with platelets also differs according to the molecular weight of fibrinogen, that with a high molecular weight supports a greater degree of ADP induced aggregation.⁷⁶² The ratio of high molecular weight fibrinogen to low molecular weight fibrinogen in plasma varies during the acute phase reactions,⁷⁶³ indicating clear variability in plasma fibrinogen which may lead to variations in platelet interactions between the circulating fibrinogen species.⁷⁶²

6.4.1.3 Clinical observations. The laboratory findings are consistent with the epidemiological associations between the level of blood fibrinogen and the measure of platelet aggregability.^{331,631,760,762} A positive correlation between plasma fibrinogen and platelet aggregate formation is measurable in patients with both non-vascular and vascular disease. Although this may be due to a common association with illness, the increased platelet aggregate level is reduced following an infusion of anicrod, a non-specific defibrinating agent.⁶³¹

Given the close association between fibrinogen and platelet aggregation briefly described above, this section evaluates the relationship between plasma fibrinogen and the platelet function test carried out in the case-control groups.

6.4.2 Methods

6.4.2.1 Patient Population. The case and control groups consisted the young males defined in Chapter 3.

6.4.2.2 Laboratory measurements, platelet function tests and statistical analysis. These methods have been described previously in Chapter 2.

6.4.3 Results

6.4.3.1 Correlations. The only association between plasma fibrinogen levels and platelet function measures in the case group was a positive association between the level of fibrinogen and the amount of TXB2 produced in clotted whole blood (Table 6.8). In the control group, there was a negative association between fibrinogen levels

and the rate of platelet aggregation in response to ADP and adrenaline, and a positive relationship between fibrinogen level and LT50ADP value (Table 6.8).

6.4.4 Discussion

A relationship between plasma fibrinogen levels and aggregation responses with the aggregating agents adrenaline and ADP was observed in the control group which is inconsistent with the laboratory findings of others.⁷⁵²⁻⁷⁵⁴ This difference may also be related to the presence of platelet activation during blood collection which is in part influenced by the blood fibrinogen level and results in less platelet aggregation *in vitro*.

The group with CHD did not show this relationship. In this group, the plasma fibrinogen appears to have a greater relationship with arachidonic acid metabolism and/or thrombin induced aggregation, given the relationship with the production of TXB2 in clotted whole blood.

Once again, since this study is an observational evaluation, the mechanism for these differences can not be determined. The results do, however, provide a stimulus for evaluating potential causes for such differences. For example, it is possible that the molecular structure of fibrinogen in these two groups may differ since fibrinogen with a high molecular weight is more responsive to ADP-induced aggregation.⁷⁶² Moreover, the beta-fibrinogen gene locus appears to be associated with an increased risk of peripheral atherosclerosis.⁷⁶⁴ Another explanation may relate to the effect of blood fatty acid concentrations on fibrinogen and/or platelet aggregability since free fatty acids may play a role in the synthesis of fibrinogen.²⁰⁸

6.5 CONCLUSION

From the associations described in this Chapter, and the potential interaction between *in vitro* and *in vivo* platelet function and plasma fibrinogen, the importance of controlling for plasma fibrinogen as a potential confounding factor in any analysis of platelet function and the association with CHD, is clearly mandatory.

Table 6.1. Patient characteristics.

Fibrinogen (mg/100ml)	314 +/- 97
Cholesterol (mmol/l)	5.62+/- 0.96
Triglyceride (mmol/l)	1.98+/- 0.94
HDL cholesterol (mmol/l)	0.98+/- 0.25
Smokers (past & present)	74 (81%)
Packyears smoking	37 +/- 24
Number currently smoking	2
Hypertension diagnosed	17 (19%)
Weight (kilograms)	80.3 +/- 9.1
Time since last AMI (days)	240 (35 to 2990)
creatinine (mmoles/l)	94 +/- 26
Number stenoses > 50%	3.3 +/- 2.3
Number stenoses > 70%	2.8 +/- 2.2
Number total occlusions	0.9 +/- 0.9
Coronary atheromatous score (CAS)	20.5 +/- 12.8
Coronary stenosis score (CSS)	30.9 +/- 22.1
Left ventricular score (LVS)	2.7 +/- 2.4

All values expressed as mean +/- SD or number (percentage).

Table 6.2. Univariate correlations with fibrinogen and age (n=91).

	fibrinogen	age
Age (years)	0.21 *	
Packyears (n=74)	0.21 *	0.07
Cholesterol	0.15	0.19 *
HDL cholesterol	-0.34 **	-0.36 ##
Triglycerides	0.25 *	0.07
Systolic BP	0.15	0.29 *
Number of infarcts (n=74)	0.22 *	-0.13
Days from infarct (n=74)	0.06	
Blood sugar	0.05	
Fibrinogen		0.21 *
Number of stenoses >50%	0.21 *	0.22 *
Number of stenoses >70%	0.27 #	0.31 **
Number of occlusions	0.28 #	0.03
CAS	0.14	0.38 ##
CSS	0.32 **	0.28 **
LV Score	0.31 **	0.07
Normal Segments	-0.15	-0.32 **
Segments with diffuse disease	0.16	0.23 *
Arteries with distal disease	0.17	0.18 *
Segments with calcification	0.14	0.40 ##

* p<0.05 # p<0.01 ** p<0.005 ## p<0.001

Table 6.3. Multiple regression analysis of fibrinogen for associated variables.

Variables	B	Rsqr	SE	p value	95% CI of B
HDL	-1.633	0.130	0.597	0.009	-2.832/-0.433
Age	82.751	0.263	27.868	0.005	26.747/138.754
Packyears	1.635	0.366	0.585	0.007	0.460/2.810
CSS	1.221	0.419	0.589	0.044	0.037/2.405

B=regression coefficient, Rsqr=coefficient of determination

SE=standard error of B, CI=confidence interval

Table 6.4. Comparison of risk factors between patients with and without left ventricular wall motion abnormalities.

Wall Motion	Normal (LVS=0) n=23	Abnormal (LVS>0) n=68
Fibrinogen (mg/100ml)	266 +/-67	329 +/-99 **
Cholesterol (mmol/l)	5.92+/-0.89	5.51+/-0.97
HDL cholesterol (mmol/l)	1.08+/-0.30	0.94+/-0.23
Triglyceride (mmol/l)	1.80+/-0.81	2.04+/-0.99
Systolic BP (mmHg)	117 +/-11	125 +/-13
Packyears (n=18 & 56)	25 +/-22	41 +/-24 #

* mean +/- SD, # p<0.02, ** p<0.001, BP=blood pressure

Table 6.5. Univariate associations with fibrinogen in the case-control study groups.

	CONTROLS	CASES
Age (years)	0.21 p=0.09	0.21 p=0.07
Packyears	0.01	0.17
Cholesterol	0.22 p=0.08	0.01
HDL cholesterol	-0.11	-0.27 p=0.03
Triglycerides	-0.13	0.02
Apoprotein A	-0.08	-0.13
Apoprotein B	0.37 p=0.02	0.12
BSL	-0.13	0.39 p=0.004
HbA1C	-0.10	0.30 p=0.02
Weight	0.04	0.33 p=0.01
Height	0.04	0.01
BMI	0.01	0.40 p=0.002
Scapular	0.06	0.41 p=0.005
Haemoglobin	0.27 p=0.04	0.28 p=0.03
Haematocrit	0.29 p=0.03	0.18
WCC	0.07	0.06

Pearson correlation coefficients, See Table 6.1 for abbreviations.

Table 6.6. Multiple regression with fibrinogen as the dependent variable and those variables with a univariate association as the independent variables.

Independent Variable	B	SE B	Beta	Rsqr	Sig
A. CONTROL GROUP					
Haemoglobin	1.6948	0.6820	0.4251	0.1807	0.0192
B. CASE GROUP					
Scapular	0.6732	0.2020	0.5135	0.2637	0.0022

See Table 6.1 for abbreviations.

Table 6.7. Multiple regression with fibrinogen as the dependent variable and those variables with a univariate association as the independent variables as well as pack-years.

Independent Variable	B	SE B	Beta	Rsq	Sig
A. CONTROL GROUP					
Haemoglobin	1.6948	0.6820	0.4251	0.1807	0.0192
B. CASE GROUP					
Scapular	0.6732	0.2020	0.5135	0.2637	0.0022

B=regression coefficient, Rsq=coefficient of determination. SE=standard error, Beta=Beta coefficient, sig=p value. Scapular=scapular skin fold thickness.

Table 6.8. Univariate associations between fibrinogen and platelet function measures.

	CONTROLS	CASES
Platelet count	-0.07	0.05
Platelet Count Ratio	0.15	-0.16
Serum TXB2	-0.16	0.35 #
Plasma BTG	0.06	-0.13
MPV	-0.15	0.05
PRPPC	-0.08	-0.09
LT50ADR	0.15	-0.08
RADR	-0.36 *	0.14
LT50ADP	0.33 *	-0.02
RADP	-0.33 *	0.01
LT50COL	0.08	-0.11
RCOL	-0.16	-0.27
LAGCOL	-0.06	-0.19

Pearson correlation coefficients,
See Tables 6.1 and 6.2 for abbreviations.

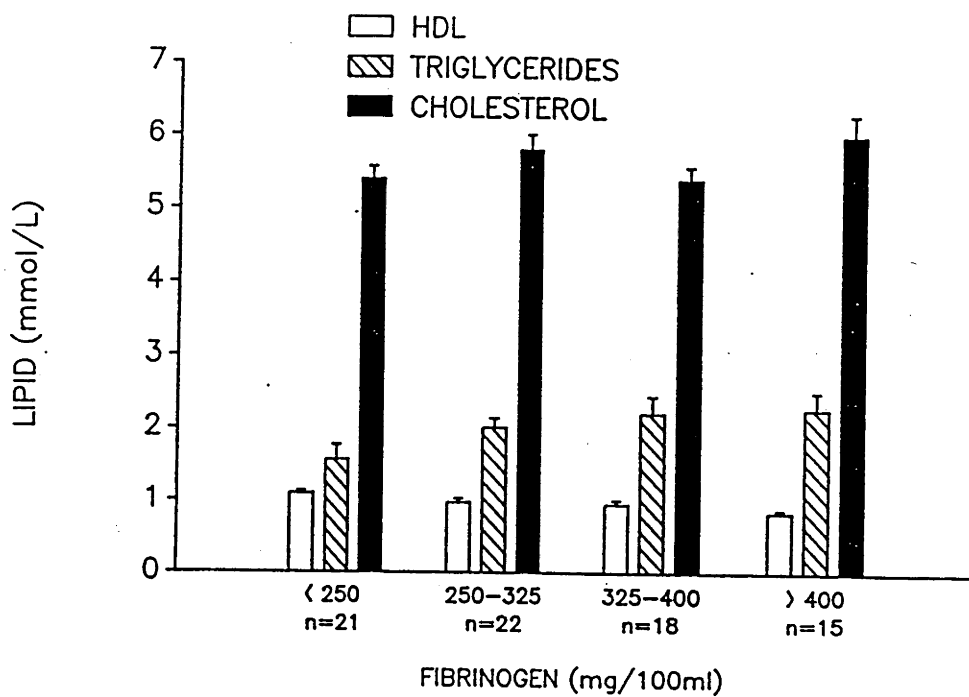


Figure 6.1. Group mean values of lipids (mean \pm SEM) for increasing categories of fibrinogen level.

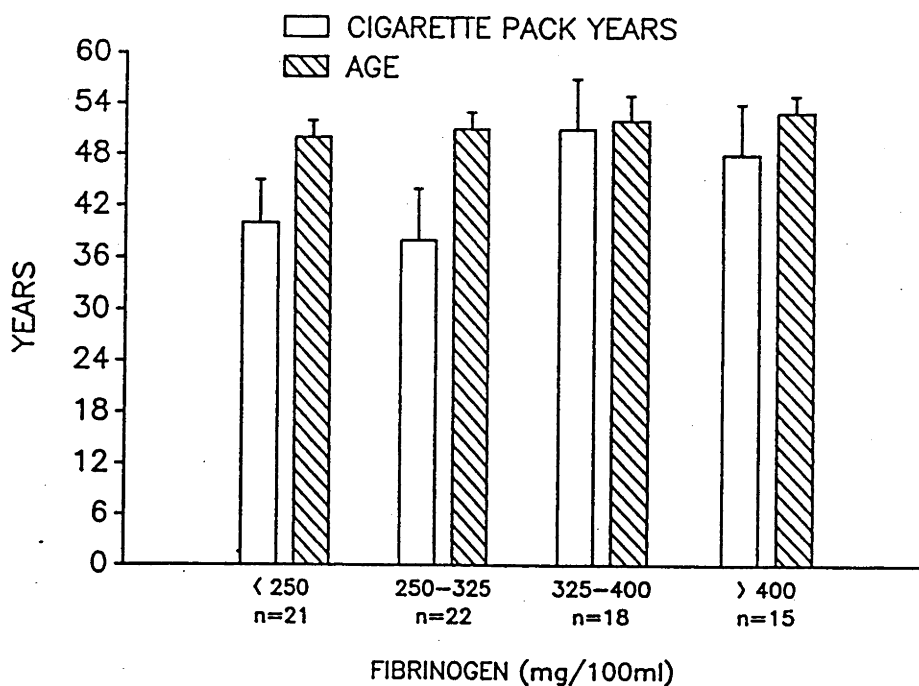


Figure 6.2. Group mean values of age and smoking (mean \pm SEM) for increasing categories of fibrinogen level.

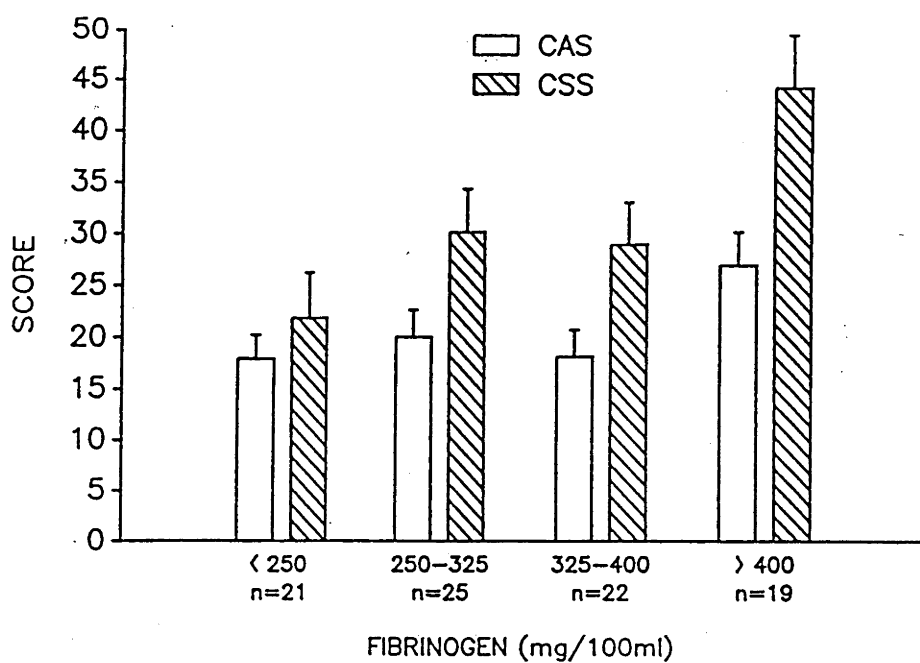


Figure 6.3. Group mean values of extent (CAS) and severity (CSS) of CAD (mean \pm SEM) for increasing categories of fibrinogen level.

CHAPTER 7

PLATELET FUNCTION AND CORONARY HEART DISEASE

7. PLATELET FUNCTION TESTS

The results presented in this chapter pertain to the hypothesis that there is an increase in platelet reactivity in males with premature CHD, and that this constitutes a measureable risk factor for the disease. The measures include MPV, PCR, BTG, plasma TXB₂, serum TXB₂ and *in vitro* platelet aggregation. The results for the different measures are presented and discussed in detail in each section of this chapter.

As previously emphasised, there appears to be no evidence of continuous activation of platelets in patients with stable CHD between acute coronary events,^{45,298} an observation supported by the results in this Chapter. Platelets are involved in the progression of atherosclerosis and in the development of thrombosis after atherosclerotic plaque rupture.²⁶¹ These events may involve circulating platelets with altered function.⁷⁶⁵ Unfortunately, the selection of platelet function tests which may be used to detect a latent but increased potential for platelet activation as a means of predicting the development of atherosclerosis, or its complications, may be inadequate for this purpose.^{765,766} The need to elucidate which platelet reactions, if any, best predict increased risk of AMI has frequently been emphasised.⁴⁷⁵ Therefore, measures of platelet aggregation require further evaluation to establish their utility for such purposes.^{767,768}

7.1 MEAN PLATELET VOLUME AND CHD

7.1.1 Introduction

7.1.1.1 Platelet size, volume and density, and platelet function. Normal circulating platelets are heterogeneous in size and density.⁷⁶⁹⁻⁷⁷¹ It has been postulated that this platelet heterogeneity is due to aging of circulating platelets. Platelets were thought to change in size, becoming smaller and lighter with aging.^{770,772} However, it would appear that circulating platelets do not change in size⁷⁷³ or density with senescence.^{774,775} Large platelets may not be younger platelets, but rather platelets with a longer lifespan.⁷⁷³ Platelet heterogeneity may result from the heterogeneity of megakaryocytes,⁷⁷⁴ which may be due to production factors in the bone marrow.^{774,776,777} Platelet volume may also be determined during thrombocytopoiesis.⁷⁷⁷ Platelets with a larger mean volume are derived from megakaryocytes with a larger mean cytoplasmic volume,⁷⁷⁸ and this appears independent of aging.^{771,779}

Platelet size and age both appear to be independent determinants of platelet function.^{771,776,780} Larger platelets, for example, are more reactive than smaller platelets *in vitro*.^{781,782} This may be due to quantitative differences based on size rather than qualitative differences due to aging and other factors.^{773,783} The response of platelets to the aggregating agents thrombin and collagen increases in proportion to their MPV.⁷⁷⁶ The release of arachidonic acid metabolites is also quantitatively related to their size,⁷⁸³ and the ability of platelets to incorporate arachidonic acid, convert it to active metabolites, and release these metabolites, is related to their volume.⁷⁸³ The ability of platelets to release unmetabolised arachidonic acid varies inversely with the MPV.⁷⁸³ There is a linear relationship between TXB₂ production and MPV.⁷⁸⁴

Furthermore, following induction of thrombocytopenia, the mean cytoplasmic volume increases in megakaryocyte populations with a higher than normal mean polidy number. Such megakaryocytes generate more TXB₂ per volume and the larger platelets derived from the large megakaryocytes are more reactive. In addition, these platelets produce more TXB₂ per unit platelet volume.⁷⁸⁴

An increase in platelet reactivity associated with increased platelet volume has been confirmed by other methods used to assess platelet reactivity.^{776,785,786} The inhibitory effects of PGI₂ on aggregation are dependent on both platelet volume and platelet count.⁷⁸⁶ This inhibitory effect decreases with an increase in platelet mass and is independent of whether it resulted from increased volume or platelet count.⁷⁸⁶ Furthermore, a decrease in the inhibitory effectiveness of PGI₂ on both aggregation and the release reaction occurs in the presence of a greater mass.⁷⁸⁶ This is also independent of whether the increased mass is due to increased platelet volume or number.⁷⁸⁶

Larger platelets appear to have both increased metabolic as well as functional capabilities. Increased glycogenolysis, glycogen and protein synthesis, and greater contents of orthophosphate and nucleotides have been noted in larger and heavier platelets.⁷⁷² Furthermore, larger platelets manifest increased clot retraction ability, greater adherence to collagen, increased aggregation with ADP and greater release of granule contents in response to ADP.^{771,781}

It is possible that these differences are due to the physical properties of different sized particles.⁷⁸⁵ Large platelets may aggregate more readily than small platelets because increased *in vitro* reactivity may reflect, not differences in platelet function, but more frequent collision of larger platelets.⁷⁸⁵ However, given the preceeding discussion, this simple physical proposal is unlikely to be the solitary explanation. Platelets separated into size dependent populations also show no differences in the rate and extent of clumping in response to ristocetin.⁷⁷⁶ Furthermore, the absolute amounts of ADP and BTG are greater within larger platelets and the amounts secreted in the release reaction have a close correlation with platelet size.⁷⁷⁶

In summary, even though it is well established that different sized platelets have different metabolic capabilities, and different physical and functional properties, the relevance of platelet volume to platelet physiology and pathology remains controversial. The cause of these differences also remains uncertain, although the quantitative differences are well established.⁷⁷¹

7.1.1.2 The relationship between platelet count and platelet size. An inverse linear relationship between platelet count and size is well documented.⁷⁸⁷ The proportion of larger platelets (volumes >8 fl) is greater in normal individuals with lower platelet counts, and there are proportionally more small platelets (<8 fl) as the platelet count increases.⁷⁸⁸ Platelet count and volume appear to be inversely but not linearly related in normal subjects.^{779,789,790} This relationship exists in patients with low or high platelet counts. Levin and Bessman found that the product of the platelet count and size varies substantially within the normal range, although the platelet mass increased as platelet count increased.⁷⁷⁹ At platelet counts less than 450 10⁹/l, any groups with a difference in platelet count of >80 10⁹/l had significantly

different volumes.⁷⁷⁹ With progressively lower counts the MPV increases. The most marked difference in MPV occurs when the platelet count is below $100 \times 10^9/l$ and there is no difference in MPV with a platelet count above $450 \times 10^9/l$. The platelet mass increases in a non-linear manner as the platelet count increases,⁷⁷⁹ although the platelet mass is constant for counts between 100 to $450 \times 10^9/l$ when correcting for splenic sequestration of larger platelets and inherent errors in electronic measurement of MPV.⁷⁷¹ In the absence of haemostatic "stress" both the platelet count and MPV remain constant over time in a given individual.⁷⁷¹

7.1.2 Methodology for the Measurement of Platelet Size

7.1.2.1 Methods available and their limitations. There is a good correlation between whole blood and PRP platelet counts, independent of MPV.⁷⁹¹ Mean platelet volumes in whole blood and PRP also correlate well.⁷⁹¹ Platelet volume has a log-normal frequency distribution in whole blood platelet populations,^{356,769,779,781,791} and the distribution curve appears to be unimodal and is skewed to the right.^{356,792} However, the log-normal distribution of platelet volumes may only be present over the range 3-15 fl.⁷⁹² There is satisfactory confirmation of a unimodal model for platelet size distributions in the range >2 fl and <20 fl.⁷⁷⁷

Unfortunately, many technical considerations, such as the full recovery of all platelets in PRP, contamination of PRP by platelet aggregates, and shape change occurring during the processing, make the evaluation of the relevance of platelet physical heterogeneity difficult when using PRP as the platelet source.⁷⁷¹ The preparation of PRP results in the loss of at least 2-5% of platelets⁷⁹¹ and possibly 20-40% originally present in whole blood.^{769,781} The necessity of total platelet isolation to avoid exclusion of platelet subsets that would lead to experimental bias and loss of important functional subgroups of platelets has been considered.⁷⁷⁰ Centrifugation does not appear to affect shape as substantiated by microscopic methods.³⁵⁶ The platelet size distribution is not manifestly influenced by particle concentration (50,000 to 400,000) or by platelet shape.³⁵⁶ Continuous density gradients may be a method of obtaining separated platelets,⁷⁷⁷ although the method is not ideal for clinical screening application.⁷⁷¹

Further problems arise because of the time dependent changes in platelet volume *ex vivo* that occur after the collection of blood. There appears to be a predictable increase early after venesection,⁷⁹¹ although this has not been a consistent finding.⁷⁸⁸ In both whole blood and PRP, the MPV can increase between 21 to 31% within five minutes of exposure to EDTA.⁷⁷⁹ This increase may be as low as 2% or possibly up to 46%.⁷⁹³ A further 10 to 16% increase in volume occurs over the subsequent two hours and following this the volume remains constant.⁷⁷⁹ During this period the platelet count changes less than 5%. There is little difference between the MPV measurement of whole blood and PRP. Reproducibility studies over a period of three months have demonstrated only a 6% variation in MPV during sequential observation.⁷⁷⁹ An increase in the MPV caused by EDTA^{779,793} may be prevented by using sodium citrate and PGE1 as the anticoagulant.⁷⁹⁴

Methodology, anticoagulation and storage time all influence the MPV.^{793,795} Measurement systems for determining MPV can give different results with very little

change in platelet count.⁷⁹³ Different anticoagulants, different concentrations of the same anticoagulant⁷⁹⁵ and different storage times in the same anticoagulant^{793,795} all alter the MPV. Alteration in the shape and the percent distribution of various shaped platelets occur at all steps in platelet isolate preparations. The method of venepuncture is of initial importance and smaller gauge needles, prolonged withdrawal and simply the insertion of a needle into a vein and application of a tourniquet affect the percentage of discocytes present.⁷⁹⁶ Other variables involved include the type of anticoagulant, temperature and pH, fixation method and 'washed' platelets.³⁵⁶ All these factors alter the distribution of platelet shape.^{781,796}

As a result of these and other limitations, measurement of platelet heterogeneity has not achieved general practical clinical application.

7.1.2.2 Electrical cell sizing. Changes in MPV can arise from differences in the distribution of platelet morphology, particularly when using electrical cell sizing (Coulter counter techniques). Existing methods for measuring MPV tend to overestimate the size of small platelets and underestimate the size of large platelets.⁷⁷¹ A decrease in discoid shaped platelets and an increase in ellipsoid platelets with pseudopods results in an apparent increase in MPV of 10 to 30%.⁷⁹⁷ This is thought to be due to the shape change and is not necessarily related to an absolute volume change.⁷⁹⁶ However, the good agreement in values for MPV obtained by different laboratories using the Coulter counter technique suggests that the variation in techniques for both the isolation and measurement of platelet physical properties may not be of major importance in normal subjects.^{791,796}

Advances in electrical cell sizing have made the MPV a readily available parameter in most clinical laboratories.⁷⁹⁸ When particle shape, electrolyte resistance, electronic amplification and capillary aperture diameter are kept constant, the Coulter counter produces electrical impulses whose amplitude is proportional to the volume of the individual particles passing through its aperture.³⁵⁶ Calibration of the instrument is achieved using fixed size latex particles, a method which does not take into account the effect of shape change. There usually is no correction for particle concentration despite demonstration of an inverse relation between size and platelet count. However, the justification for not correcting for these factors when using the Coulter counter to measure MPV³⁵⁶ is not universally accepted.^{779,789,790}

The Coulter counter model S-Plus counts platelets in whole blood by developing a histogram, which is normally skewed to the right of particle volumes in the range 2-20 fl. The instrument's computer software programme extrapolates this curve to a smooth log-normal curve and in doing this artificially includes particles >20 fl and <2 fl. The platelet count subsequently reported includes all particles within the extrapolated curve. The MPV is calculated by integration of the mean of all particles under this curve.^{789,791} If a trough is noted in the histogram at particle volumes less than 20 fl then that volume is used as the upper limit for that particular specimen. If the histogram can not be smoothed to a log-normal curve, no MPV and possibly no platelet count will be reported.⁷⁸⁹

7.1.2.3 Alternative methods. Microscopic measurements of platelet diameters have been performed on peripheral blood smears of platelets in citrated blood or

with PRP prepared from blood anticoagulated with citrate or EDTA.³⁵⁶ The volume is then calculated from the measured diameters. This method is slow, labour intensive and not suitable for general clinical application. The MPV distribution obtained by this method correlates with that obtained by electronic sizing and counting,^{356,780} although it is probably less sensitive to volume changes.³⁵⁶ The distribution of platelet diameters from smears and the apparent volume distribution from the Coulter counter are both comparable with a log-normal distribution.^{356,799}

Another method of separating platelets according to size independent of density employs the principle of Counterflow centrifugation (centrifugal force opposed by a flow force to separate particles).⁷⁸² Other methods of separation of different sized platelet fractions have relied on discontinuous density gradients.^{769,770,772} on continuous density gradients⁷⁷⁷ and also fluorescent activated cell sorting.⁷⁷⁷ The separation of platelets into subfractions for measurement of MPV introduces the increased possibility of introducing artifacts (activation, shape change and even possibly a decrease in density).

The applicability of automatic electronic cell sizing for whole blood appears to be the preferable clinical screening method for measuring MPV, accepting the limitations of the method. However, it must be emphasised that the MPV is but one aspect of the morphological heterogeneity manifested by circulating platelets.⁷⁷¹

7.1.3 Platelet Size and Volume in AMI and CHD

Larger platelets, as measured by an increased MPV, have been reported to be more active when assessed by changes in platelet aggregation, survival time or the release reaction.^{771,772,776} An increased platelet volume has been observed in patients with AMI.^{800-803,794} Furthermore, increases in platelet volume has been documented six weeks⁸⁰² to three months after AMI.⁵⁴ Using different methodology, a number of other studies have also demonstrated a higher MPV in patients with AMI compared to controls.^{804,805} Platelet size may also be a predictor of recurrent MI and death,⁸⁰⁶ although it previously was thought not to provide prognostic information.⁸⁰¹ Increased platelet volume has also been found in patients with chronic stable angina.⁵⁴ This is not a uniform observation.⁸⁰⁴

The cause of a larger MPV in patients with AMI is uncertain, is unassociated with diabetes or smoking⁸⁰¹ and other known risk factors for CAD such as BP, blood lipids, fibrinogen, WCC or plasma viscosity.⁸⁰⁶ The observation that there is an overall increase in the normal distribution curve of platelet volume mitigates against an increased production and consumption of a subpopulation of platelets due to AMI.⁸⁰² Bone marrow megakaryocytes volumes are larger than normal in individuals dying of sudden cardiac death and in association with AMI.⁷⁹⁴ There is no significant difference between the platelet cytoplasmic volume distribution for patients with AMI or sudden coronary death.⁷⁹⁴ Lower platelet counts have been documented in patients with AMI compared with controls,⁸⁰⁵ although this remains controversial.⁸⁵ Furthermore, patients who have had AMI, and who have a large MPV, may also have a lower platelet count.⁸⁰⁴

Methodological problems related to measuring MPV in infarct patients may account

for these conflicting observations described.⁷⁷¹ Activated platelets undergo shape changes that can cause artifactual changes in volume in a resistive particle counting system,⁷⁹⁷ and platelets are activated in AMI.⁴⁵ This problem can be minimised by using PGE1 with sodium citrate to inhibit platelet shape change. However, a large MPV and a low platelet count have still been observed in individuals with AMI even when these anticoagulants are used.⁷⁹³ Furthermore, if there was simply an artifactual increase in the size of platelets, it would be accompanied by a change in the skewedness of the volume distribution.

An additional population of platelets may contribute to the increased MPV observed in patients with CHD. A bimodal distribution in the MPV has been noted in an infarct group,⁸⁰⁴ the lower volume group being similar to a normal control group. In this study, those individuals with a larger MPV also had lower platelet counts,⁸⁰⁴ and this observation was recently confirmed.⁸⁰⁵ Alternatively, this apparent bimodal distribution may be due to a deficit of small platelets, being an effect rather than a cause of AMI.⁸⁰⁷ A group was followed for 18 months after AMI and the distribution curves at this time did not differ significantly from those of a control group.⁸⁰⁷ Retrospectively plotting the platelet volume distribution from data measured at the initial time of the infarction indicated the possibility of a deficiency of platelets in the range of 5-12 fl, there being no difference above 12 fl. The difference had disappeared by 18 months. This observation supports the concept that the high MPV is due to a deficiency of small platelets and is possibly an effect rather than cause of AMI. Indeed, others have provided supporting evidence for the postulate that a high MPV after an AMI is due to consumption of small platelets.⁸⁰⁸ Others argue that alterations in the entire megakaryocyte-platelet-haemostatic axis precedes the MI.^{794,805,806} Increased total aggregated, irreversibly aggregated, reversibly aggregated platelets, and average platelets per aggregate are increased after an AMI.⁸⁰³ These changes are especially marked in patients later developing cardiac complications.⁸⁰³ Moreover, men who have a fatal or further non-fatal MI within 2 years of an initial MI have a greater MVP 6 months after the first MI than survivors who have no further events.⁸⁰⁶

7.1.4 Methods

See Chapter 2, Chapter 8 and Appendix 1 for a description of the methods and standardisation.

7.1.5 Results

7.1.5.1 Group comparisons. A significant difference in the values for MPV was noted between the case and control groups. The MPV in the case group was significantly larger (9.4+/-0.9 fl, median 9.2fl, min=7.0fl, max=12.4fl) than in the control group (8.9+/-0.9 fl, median=8.8fl, min=6.8fl, max=11.3fl).

7.1.5.2 Correlations with blood cell indices, CHD risk factors and platelet function tests. In the control group, the MPV had a significant inverse linear correlation with the platelet count in blood and the platelet count in PRP (PRPPC) (Table 7.1). There was also a borderline inverse relationship with the WCC, the cholesterol level and the PCR (Table 7.1).

For the case group, the MPV inversely correlated with the platelet count, the PRPPC, Hb, Hc, triglyceride, HDL cholesterol and serum TXB2 levels (Table 7.1). No relationship was observed with the plasma BTG level (Table 7.1) or consistently with any of the measures of platelet aggregation in either group (Table 7.2).

7.1.5.3 Correlations with plasma fatty acid. Since fatty acids have been shown to influence platelet function and membrane content, the relationship of the MPV to the plasma fatty acids was evaluated. A positive relationship between the MPV and oleic and eicosapentaenoic acids, and negative linear correlation with palmitic was present in the control group (Table 7.3). This differed from that observed in the case group. A positive correlation between MPV and docosahexaenoic acid, and negative correlation with stearic and eicosatrienoic acids was observed in the case group (Table 7.3).

7.1.5.4 Regression analysis. To evaluate the independent predictive value of those variables linearly correlating with the MPV (platelet count, Hb, Hc, WCC, cholesterol, triglyceride, HDL cholesterol level, palmitic (C16:0), stearic (C18:0), oleic (C18:1w9), eicosopentanoic (C20:5w3) and docosohexanoic acids (C22:6w3), they were entered into a multivariate regression model. In the case group, docosohexanoic acid, HDL cholesterol and stearic acid were independent predictors of MPV. In the control group, the platelet count and palmitic acid only remained independently predictive of MPV (Table 7.4).

7.1.5.5 Analysis of covariance. In view of the difference in the group means for the MPV and the differing correlation between the MPV and variables between the groups, the effect of these variables (platelet count, Hb, Hc, WCC, cholesterol, triglyceride, HDL cholesterol level, C16:0, C18:0, C18:1w9, C20:5w3 and C22:6w3) was measured by analysis of covariance to obtain an adjusted mean. When the effect on the variance of these variables was removed, the adjusted means of the MPV for the case and control groups was 7.1fl and 7.3fl respectively. The statistics for this analysis are described in Table 7.5.

7.1.5.6 Coronary angiography. The MPV in the CHD group was related to both the disease severity and to the extent of coronary atherosclerosis measured angiographically (Table 7.6).

7.1.6 Discussion

The increase in MPV in the CHD case group is consistent with observations made in a number of other studies.^{54,802,804-806} Furthermore, the increase in MPV in the CHD group is associated with a lower platelet count, again, a finding in common with previous reports.^{787,804,805} The difference between the two groups is unlikely to be due to technical considerations affecting the measurement of the MPV, although many difficulties exist.^{356,769,771,779,781,788,791,793,795} Exactly the same methodology was applied to the collection, processing and measurement of the MPV in both groups. Indeed, since current methods overestimate the size of small platelets, and underestimate the size of large platelets,⁷⁷¹ the reported differences in the MPV may be an underestimate. In addition, the reproducibility of the measurement of MPV using the Coulter Counter technique is very good, with a low intra- and inter-labora-

tory error.^{796,789} Thus, it would appear that patients with CHD and a previous AMI have a platelet population with a larger MPV. The validity of the observation is supported by the consistency of the observation with that in the literature.^{54,803-806}

Although there appears to be little doubt that the MPV is persistently greater in patients with CHD months after AMI,^{54,802,806} observations from studies of stable CHD without infarction have been equivocal.^{54,804} The cause of the difference has not been elucidated by this study. Being a cross-sectional study, it does not provide information about differences due to selective consumption of small platelets,⁸⁰⁸ aging^{770,772} or at thrombocytopoiesis.^{774,776,777} Another consideration in the possible relationship between platelet volume and AMI is that the case group were all clinically stable, and therefore, it would be unlikely that differences were related to ongoing coronary thrombosis. Moreover, other studies have also documented larger platelets in patients six weeks⁸⁰² and three⁵⁴ and six months⁸⁰⁶ after AMI. Nevertheless, as will be discussed, the MPV is influenced by different factors within the blood in each group, particularly the plasma fatty acid content. The lack of an association with smoking and blood glucose levels is well documented.⁸⁰¹

The observation that the platelet count is inversely proportional to the MPV in this study has also previously been well documented.^{779,787,789,790} To ensure that any measured differences in the MPV between groups are valid, adjustment for the platelet count is recommended.⁷⁸⁹ This was undertaken using analysis of covariance to obtain an adjusted mean which takes into account a number of variables that may have resulted in the difference. Blood lipoproteins and fatty acids have been clearly demonstrated to influence platelet function, including the platelet membrane content. Therefore, since such plasma factors may have a potential influence on the platelet volume, those variables related to MPV were also entered into the analysis of covariance. This analysis showed that the adjusted MPVs in the two groups did not differ, indicating that those factors associated with the MPV in blood may have caused the observed difference.

Previous reports have documented increased *in vitro* reactivity related to platelet size.^{776,781,782} However, in both our population groups, the MPV (measured in whole blood) was unrelated to *in vitro* measures of platelet aggregation (measured in citrated PRP). This negative result may be due to the loss of larger platelets during the preparation of PRP by centrifugation. As a result, the cell population used *in vitro* may be different to the *in vivo* population, with a bias toward smaller lighter platelets in the supernatant than that in the centrifuged blood used in the aggregation studies. A greater percentage of larger platelets may be removed during centrifugation, resulting in aggregation being less influenced by platelet size, with a consequent dilution of a measurable relationship between aggregation and MPV. This may not be important however, since there appears to be little difference in the MPV between platelets in whole blood and in PRP.⁷⁷⁹ Nonetheless, at least 2-5%,⁷⁹¹ and possibly 20-40%,^{769,781} of circulating platelets may be lost in the preparation of PRP.^{769,781,791}

A linear relationship between MPV and TXB2 production has been observed.⁷⁸⁴ The opposite relationship was observed in the present study, and only in the case group, despite the fact that the CHD group produced more TXB2 in clotting whole blood.

than the control group. Larger platelets may have more TXB2 synthetase resulting in the ability to produce more TXB2,⁷⁸⁴ which may explain the greater amount of TXB2 produced during clotting in the CHD group. Alternatively, since larger platelets are potentially more reactive than smaller platelets,^{771,776,781,783,784} the circulating larger platelet may become "exhausted", resulting in less reactivity *ex vivo* and lower maximum TXB2 production during clot formation.

The MPV has a univariate correlation with the number and severity of angiographic defined stenotic lesions and less so with the extent of disease. This association may be causative, resulting in an increased risk of CHD events, as has been documented.⁸⁰⁶ Alternatively, the association may arise because the MPV is a marker indicating the presence of a pre-thrombotic state.⁸⁰⁵ Another explanation may be that the increased MPV results from the CHD event, such as a AMI.⁸⁰³

7.1.7 Summary

1. The MPV in young males with CHD was greater than in normal control subjects.
2. An increase in MPV was present in individuals with CHD more than 3 months after an infarct.
3. The higher MPV occurred without evidence of ongoing *in vivo* platelet activation.
4. The MPV in the group with CHD was related to disease severity and also to the extent of atheroma within the coronary arteries. This relationship with angiographically defined CAD would indicate that the observation is unlikely to have occurred by chance.
5. The MPV is therefore an identifiable risk factor in young males with established CHD supporting similar conclusions made by others.⁸⁰⁶ That is, there is a statistically significant difference between a group with disease compared to a group without disease, without there being grounds for inferring causality. As with most risk factors, the large overlap between individuals in both groups prevents the measurement of the MPV from being a clinical useful parameter for disease identification or assessment of severity, at present.
6. Other interacting influences, namely the content of plasma fatty acid, may have resulted in the difference observed. Hence the increased MPV may be only a marker for another pathophysiologically abnormality present to a greater degree in the CHD group.

Table 7.1. Linear correlations with the MPV for controls and cases.

Variable	Control	Case	Variable	Control	Case
DOB	0.01	-0.11	CHOL	-0.26 *	-0.01
PACKYR	-0.06	-0.26	TRIG	-0.18	-0.29*
ETOHGM	-0.28 *	-0.10	HDL	-0.05	-0.29*
WT	-0.14	-0.16	APOA	0.03	0.16
HT	-0.01	-0.03	APOB	-0.13	-0.04
BMI	-0.14	-0.14	Hb	-0.01	-0.32 #
TRICEP	0.01	0.10	Hc	-0.01	-0.30 *
SCAP	0.01	0.16	WCC	0.23	-0.09
SBP	0.04	-0.02	MCV	-0.05	-0.11
DBP	-0.01	-0.08	PRPPC	-0.29 *	-0.45 #
BSL	-0.01	-0.14	PCR	-0.24	-0.16
HbA1C	-0.19	-0.02	SerTXB	0.03	-0.37 **
FIBRIN	-0.15	0.05	BTG	-0.17	0.07
URATE	-0.13	0.02	PC	-0.38 #	-0.30 *

DOB=date of birth, packyr=number of years smoking the equivalent of 20 cigarettes per day, ETOHGM=portions of alcohol per day, WT=weight, HT=height, BMI=body mass index, TRICEPS=triceps skinfold thickness, SCAP=scapula skinfold thickness, SBP=systolic BP, DBP=diastolic BP, BSL=blood glucose level, HbA1C=glycosylated Hb, FIBRIN=fibrinogen level, URATE=urate level, CHOL=blood cholesterol, TRIG=triglyceride level, HDL=HDL cholesterol, APOA=apoprotein A1, APOB=apoprotein B, Hb=haemoglobin, Hc=haematocrit, WCC=white cell count, MCV=mean red blood cell volume, PRPPC=platelet count in PRP, SerTXB2=serum thromboxane B2, BTG=beta-thromboglobulin, PC=platelet count.

Table 7.2. Linear correlations for controls and cases with MPV and measures of *in vitro* platelet aggregation.

Variable	Control	Case	Variable	Control	Case
LT50ADR	-0.08	0.06	RADR	-0.08	-0.20
LT50ADP	-0.14	0.02	RADP	-0.01	-0.07
LT50COL	-0.14	0.25	RCOL	-0.01	-0.05
LAGCOL	-0.06	0.17			

LT50=lag time to 50% of maximal alteration in light transmission with aggregation by agonists adrenaline (ADR), collagen (COL) and ADP.

R=maximum rate of change in light transmission with aggregation for each of the agonists ADR, COL and ADP.

LAG=the lag phase before shape change with collagen induced aggregation.

See section 2.6 for abbreviations.

*<0.05 #<0.01 **<0.005 ##<0.001

Table 7.3. Correlations for controls and cases with MPV and plasma fatty acids.

Fatty Acid	Control	Case	Fatty Acid	Control	Case
Myristic C14:0	-0.12	0.14	Eicosenoic C20:1n9	-0.08	-0.08
Palmitic C16:0	-0.34#	-0.14	Eicosadienoic C20:2n6	-0.19	-0.28*
Palmitoleic C16:1n7	-0.18	-0.17	Dihomo-γ-linoleic C20:3n6	-0.18	-0.20
Stearic C18:0	-0.05	-0.25*	Arachidonic C20:4n6	-0.04	-0.15
Oleic C18:1n9	0.30*	0.13	Eicosapentaenoic C20:5n3	0.25*	-0.01
Linoleic C20:2n6	0.05	0.12	Docosapentaenoic C22:5n3	-0.06	0.03
α-Linolenic C18:3n6	0.14	-0.22	Docosahexaenoic C22:6n3	0.23	0.41**

Table 7.4. Regression analysis with MPV as the dependent variable and all parameters with a linear association entered as the independent variables for each group.

Independent Variable	B	SE B	Beta	R sq	P value
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A. Case Group (Independent variables being platelet count, Hb, Hc, WCC, cholesterol, triglyceride, HDL cholesterol, C16:0, C18:0, C18:1w9, C20:5w3, C22:6w3).

C22:6w3	0.042	0.011	0.497	0.170	0.0004
HDL	0.169	0.058	0.393	0.253	0.0059
C18:0	-0.018	0.007	-0.334	0.355	0.0144

B. Control Group (Independent variables being platelet count, Hb, Hc, WCC, cholesterol, triglyceride, HDL cholesterol, C16:0, C18:0, C18:1w9, C20:5w3, C22:6w3).

PC	-0.060	0.025	-0.337	0.134	0.0236
C16:0	-0.006	0.003	-0.313	0.231	0.0350

See Tables 7.1 and 7.3 for abbreviations

Table 7.5. Analysis of covariance with MPV as the dependent variable and Hb, Hc, PC, WCC, cholesterol, triglyceride, HDL cholesterol C16:0, C18:0, C18:1w9, C20:5w3 and C22:6w3 as the covariates (Abbreviations are in Tables 7.1 and 7.3)

(1) Tests of significance for MPV:

Sources of Variation	Sums of Squares	DF	MS	F	sig F
Within cells	303.3	40	7.58		
Regression	1471.0	11	133.81	17.4	0.000
Constant	16.57	1	16.57	0.22	0.643

(2) Regression analysis of within cells:

Covariate	B	Beta	Sig	Lower95%CI	Upper95%CI
Hb	-0.251	-0.621	0.000##	-0.372	-0.130
Hc	0.094	0.411	0.075	-0.010	0.199
PC	-0.012	-0.043	0.654	-0.065	0.042
CHOL	0.081	0.036	0.648	-0.273	0.433
TRIG	-19.260	-0.262	0.008 *	-33.304	-5.214
HDL	-0.093	-0.703	0.131	-0.214	0.029
C22:6w3	0.005	0.151	0.725	-0.023	0.033
C18:0	-0.018	-0.267	0.091	-0.039	0.003
C20:5w3	0.001	0.031	0.948	-0.013	0.014
C18:1w9	0.002	0.134	0.474	-0.004	0.009
C16:0	-0.006	-0.167	0.195	-0.015	0.003

Table 7.6. Linear correlations for controls and cases with MPV and angiographic measures of CAD.

CSS	0.27 *	No. normal segments	-0.24 *
mCSS	0.26 *	No diffuse segments	0.36 #
CAS	0.22	No. lesions >50%	0.20
mCAS	0.23 *	No. lesions >70%	0.36 #
		No. occlusions	0.10
Jenkins	0.23 *	No lesions >90%, <100%	0.25 *
LVS	0.19	No lesions >75%, <90%	0.12
CS	0.10	No lesions >50%, <75%	0.14
Collaterals	0.23 *	No lesions >25%, <50%	0.19
		No lesions >10%, <25%	0.15

CSS=coronary stenosis score, mCSS=mean CSS, proxCSS=proximal CSS, CAS=coronary atheromatous score, mCAS=mean CAS, proxCAS=proximal CAS, Jenkins=Jenkins coronary score, LVS=left ventricular score, CS=collateral score, collaterals=no arterial segments supplying collaterals, diffuse segments=diffusely diseased segments, disdistal disease=distal segments with angiographic disease.

*<0.05 #<0.01

7.2 PLATELET COUNT RATIO

7.2.1 Introduction

The PCR is a method giving an indirect quantitation of platelet aggregates in blood, and was first thought to provide a measure of *in vivo* platelet aggregates.^{325,809} Normal individuals have a PCR close to unity.⁸⁰⁹ A lower PCR occurs in various forms of vascular disease compared to a "normal" control group.³²⁵ However, a large overlap in the distribution of the PCR is present within these groups.³²⁵ In addition, there appears to be a greater variability within patients than within a normal population.^{325,584} This broad distribution limits the sensitivity of the method for use within individuals. The nature of the test and the diversity of stimuli affecting platelets makes the technique a diagnostically non-specific functional test. For the study of a population group however, the method appears to be satisfactory.^{325,327,810,811}

7.2.1.1 Limitations of the PCR measurement methods. Initially there were a number of reports that the PCR was significantly lower in patients with vascular disease and thrombotic disorders.^{325,327,810} However, doubts as to the internal reproducibility of the method and the problems of the large between group and within group variance in study populations have been raised,^{584,812} leading to concern about the validity of results using the method.^{327,812} In fact, the methodology used in this assay is highly operator dependent.^{328,813} Nevertheless, reproducible PCR measurements have been obtained with narrow normal ranges,³²⁸ as a result of changes in methodology including very accurate pipetting of blood samples or direct collection of blood into the buffered EDTA solutions, using a standardized region of the supernatant for counting, collecting a fixed volume, and reducing the amount of EDTA and formalin used.³²⁸

The technique to measure platelet aggregates is clearly susceptible to inaccuracies due to artifacts caused by sample collection and processing.^{810,812,814} Moreover, evidence has emerged that the platelet aggregates measured by this method are probably not present in the circulation of the patients.^{327,810,812,815} Indeed, there is evidence that the platelet aggregates are formed during the venipuncture.^{327,810,814} Others have found that the PCR in normal subjects is unaffected by the method of blood collection and processing when the platelet counts are measured in whole blood and not PRP.³²⁷ On the other hand, in patients with diseases associated with thrombosis, the PCR was found to be significantly lower when blood was collected through a needle and tubing when compared to when the PCR is measured in whole blood, suggesting that aggregates form during collection.³²⁷

Although it appears unlikely that platelet aggregates are commonly formed *in vivo* in vascular disease states,^{327,812} the hypothesis that the circulating platelet has the potential to express greater reactivity in certain disease states remains tenable. That is, the formation of platelet aggregates during sampling is a measure by which platelets are determined to be more reactive.³²⁷

The degree of tourniquet stasis, age, sex and the time of food intake does not appear to influence the test results.⁵⁸⁴ A very small variance within normal subjects com-

pared to that among individuals with various disease states has been documented.⁵⁸⁴ This would support the concept that there are indeed factors influencing the PCR in patients with vascular disease that are absent in normal individuals. The marked overlap between "normals" and "abnormals"³³⁴ however, makes any clinical interpretation difficult.

Other doubts about the presence of circulating platelet aggregates arise from the lack of direct evidence that platelet aggregates are present in the patient's circulation.⁸¹⁰ Circulating platelet aggregates larger than the capillary diameter would be expected to be filtered out in the microcirculation, with the possibility of impaired organ perfusion.³²⁷

7.2.1.2 Platelet count ratio in disorders involving vascular disease. In the original study in which the method was applied,³²⁵ a significantly lower PCR was found in a number of disorders involving vascular disease. The study population included patients with transient cerebral ischaemic attacks, AMI, stable angina, acute peripheral vascular insufficiency and chronic peripheral vascular disease.³²⁵ This group was compared to a "normal" and a "patient" control group. The normal control group had a PCR of 0.90 ± 0.02 and the patient control group a PCR of 0.88 ± 0.01 (mean \pm SEM). However, in the vascular disease groups, apart from those patients with a PCR less than 0.70, there was a considerable overlap in values between groups, obviously limiting the sensitivity in individuals. In addition, the nature of the method and the diversity of potential stimuli affecting the platelets during processing make the method intrinsically non-specific.

The utility of the PCR for clinical or research purposes is further confused by the failure to support the original finding of a decreased PCR in patients with chest pain, AMIs, completed strokes and transient ischaemic attacks.⁵⁸⁴ A number of research groups have observed no decrease in the PCR in individuals with stable angina compared with normal control groups.^{325,500,584,811,814,816} However, with different methods, an increase in reversibly aggregated platelets in patients with unstable angina and AMI, and an increase in irreversibly aggregated platelets in patients with stable angina compared with controls has been documented.⁸¹⁷ Scanning electronmicroscopy also reveals the presence of irreversible aggregates in the blood of patients with thrombotic episodes.⁸¹⁴ The incorporation of red and white blood cells into "platelet aggregates" was used to demonstrate more irreversible aggregates.⁸¹⁴

Increased platelet aggregates during AMI have been observed by most investigators.^{325,327,803,811,818-822} although not all.⁵⁸⁴ Patients with unstable angina have also been shown to have increased platelet aggregates.⁸¹¹ Furthermore, increased aggregates have been observed in other vascular conditions associated with intravascular thrombosis.^{325,327,819} Most studies indicate that patients with chronic stable CHD do not have a lower PCR, and by inference either circulating aggregates or hypersensitive platelets. However, those with unstable CHD do have a lower PCR which would indicate that they either have circulating platelet aggregates or that they have hypersensitive platelets.

7.2.1.3 Platelet Aggregates in Normal Subjects. Generally it is believed that aggregation does not usually occur *in vivo* in normal subjects.^{325,496,812} However,

some investigators have found platelet aggregates in normal population groups. Possibly up to 15% of platelets in the normal population may circulate as aggregates.^{584,816} Less than 10% of circulating platelets in the normal population may be present as reversible aggregates, while others have observed that about 6% of platelets circulate as aggregates which are irreversibly aggregated.⁸¹⁷ Platelets sensitised in the circulation can more easily undergo activation and aggregate upon contact with a thrombogenic surface.⁸²³

7.2.1.4 Summary. Normal groups have a broad variation in the PCR in most studies, although the PCR in normals may be narrower than originally thought when a modified technique is used.^{328,584} Attention to detail to control confounding factors in the methodology may allow differences to be more easily detected, or confirmed as being absent. Unfortunately it is difficult to compare studies since there has been a wide variation in techniques used, differences in the patient characteristics of the populations studied and small numbers in the study groups. Furthermore, when comparing a normal population group to a patient study group, the population groups have not always been well defined. In addition, drug treatment has not always been well documented.

When appropriate methods are used to calculate the necessary sample size to detect a true difference between two groups, one finds that at least 30 individuals or greater are needed to detect a significant difference in the PCR. There is no documented difference in the PCR due to age or sex in normal individuals,⁵⁸⁴ but this does not necessarily apply to the patient population with premature CHD. Therefore it is still appropriate to have relatively homogeneous study and control groups.

The wide variation in PCR found in patients with stable CHD may be related to other factors, or there may even be a bimodal distribution present. None of the previous studies have satisfactorily evaluated the PCR in patients with premature CHD. Therefore this study has evaluated such a group using methodology that has been demonstrated to produce a narrow range for the PCR in a normal population group.³²⁸ Strict controls to limit confounding methodological factors influencing aggregate formation were applied.

7.2.2 Methods

The PCR was determined by a modification of the method of Wu and Hoak³²⁵⁻³²⁸ and is described in Chapter 2.

7.2.3 Results

7.2.3.1 Group comparisons. The case group had a significantly lower PCR than the control group (0.81 ± 0.14 versus 0.87 ± 0.08 respectively, $p < 0.05$), although there was a large overlap in the values for both groups.

7.2.3.2 Correlation with blood cell indices and CHD risk factors. In the case group, the PCR did not correlate consistently with any of the major risk factors, although a significant but weak correlation existed with scapular skinfold thickness (Table 7.7). In addition, the PCR did not correlate with any of the blood cell indices

except for a negative correlation with the WCC in both groups (Table 7.7). However, in the control group there was a positive association between the PCR and the approximate amount of alcohol regularly used, as well as with the blood glucose level (Table 7.7).

7.2.3.3 Correlation with other platelet function tests. The PCR had a borderline relationship with measures of *in vitro* aggregation induced by collagen (Table 7.8). The more reactive that platelets were *in vitro*, the more that platelet aggregates were formed during blood collection in both groups, the PCR being lower. In the control group, the greater the serum TXB2 production the more that platelet aggregates formed during blood collection, a circumstance which was not present in the case group (Table 7.7).

7.2.3.4 Correlation with plasma fatty acids. Some plasma FA had a significant correlation with the PCR in the case group only. Palmitic acid (C16:0) positively correlated with the PCR whilst oleic (C18:1w9), docosapentanoic (C22:5w3) and docosahexanoic (C22:6w3) acids negatively correlated with the PCR (Table 7.9). In the control group, alpha-linolenic acid (C18:3w6) had a positive association, and C22:5w3 had a negative association with PCR, both of which were of borderline significance (Table 7.9).

7.2.3.5 Regression analysis. Those variables which may have influenced the PCR or have had a significant correlation with the PCR were entered into the step-wise regression model to determine the most predictive variable for the PCR (Table 7.10). In the case group, only oleic acid (C18:1w9), retained a significant predictive value for the PCR, whereas in the control group only the blood glucose level and the approximate alcohol intake retained a significant (and opposite) association with the PCR (Table 7.10).

7.2.4 Discussion

As observed in other studies, the normal group had a narrower range of values for the PCR than the group with CHD.^{328,584} However, the observation of a lower PCR in the case group compared to the normal control group differs from some previous studies,^{325,584,816} although not all.⁸¹⁷ These other studies evaluated older and more heterogeneous CHD groups. Our results infer that platelets from males with premature CHD are more likely to form platelet aggregates during blood collection. Nevertheless, a systematic bias due to collection procedures cannot be excluded^{810,812,814} since the method is highly operator dependent.^{328,813}

Whether circulating platelet aggregates are present or absent *in vivo* in normals is controversial.^{325,496,812,816,817} Our normal group did not have a PCR of unity, indicating the presence of aggregates in the blood samples. Most available evidence would indicate that these aggregates formed during the blood collection.^{327,810,812,814,815} The case group had more platelet aggregates in the blood samples collected. This study was not designed to determine when such aggregates appeared, *in vivo* or during the blood collection.

The possibility that the PCR is a measure of *ex vivo* platelet reactivity can be support-

ed, although not proven, on a number of grounds. Platelets from normal subjects are less influenced by blood collection methods than platelets from patients with vascular disorders,³²⁷ there is a smaller variance for the PCR in normal subjects³²⁸ than in patients with vascular disorders,^{325,584} platelets sensitised in the circulation more readily undergo activation and aggregation,⁸²³ and the PCR is decreased in conditions where *in vivo* platelet activation has been proven to occur.^{325,818-820} In the present study, a modest relationship between the PCR and *in vitro* measures of platelet aggregation, but not *in vivo* measures of platelet activation, was demonstrated. These observations further support the proposition that the PCR is a measure of *ex vivo* platelet reactivity, rather than a measure of the presence of *in vivo* platelet aggregates.

Specific factors, such as age and sex, which are associated with *in vitro* aggregation,³³¹ have not been shown to be associated with the PCR.⁵⁸⁴ Furthermore, we have shown no relationship between the fibrinogen level and the PCR, fibrinogen being a factor associated with *in vitro* aggregation measures.³³¹

A similar relationship has been documented between the amount of alcohol used and the PCR, as well as a borderline relationship with previous tobacco intake for normal subjects with the *in vitro* aggregation results.³³¹ In fact, the relationship with alcohol in the normal control group maintained a predictive association independent of the other variables. It is not possible to make any interpretation concerning the influence of alcohol in the case group since a major proportion of the subjects had changes in their pattern of alcohol use (see Chapter 5). The results, however, support the proposal of a favourable effect of alcohol on platelet function, and that the changes could conceivably reduce thrombotic CHD events.¹⁶⁴

Abnormalities of platelet function in vascular disorders have been consistently demonstrated and by various methods of evaluation.^{46,141,824-826} Increased platelet sensitivity may be a factor in the development of vascular disease in diabetic patients.^{141,824} Although a relationship between the PCR and measures of glucose metabolism has not been systematically evaluated in a normal population, a relationship between decreased PCR and diabetes has been documented.⁸²⁵ Our study groups were selected on the criteria of not having diabetes or overt glucose intolerance. Nevertheless, in the normal group, but not the CHD group, an independent association was demonstrated between the PCR and the fasting blood glucose level. The significance of this observation is uncertain at present and may simply be due to chance from multiple comparisons.

7.2.5. Summary

1. A difference between the control and case groups has been demonstrated, the PCR being lower in young males with CHD, most of whom have had a previous AMI.

2. This difference in the PCR (a functional measure of platelet reactivity) appears to be predicted by different factors in the two groups. Whether this is due to intrinsic platelet factors, influences within the blood and/or environmental effects, remains uncertain.

Table 7.7. Correlations between mean PCR, risk factors for CHD and platelet function measures in the control and case groups.

	Controls	Cases		Controls	Cases		Controls	Cases
DOB	-0.17	-0.18	CHOL	0.11	0.18	TXB2	-0.28*	-0.18
PACKYR	-0.27	0.09	TRIG	-0.07	-0.19	BTG	0.10	0.03
ETOHGM	0.35#	0.05	HDL	0.20	0.19	MPV	-0.24	-0.16
WT	0.02	0.15	APOA	0.17	0.16			
HT	-0.03	0.12	APOB	-0.02	0.12	Hb	0.14	0.02
BMI	0.04	0.08	BSL	-0.57*	-0.17	Hc	0.19	-0.01
TRICEP	-0.10	-0.14	HbA1C	0.21	-0.15	MCV	0.38#	0.13
SCAP	-0.10	-0.29*	FIBRIN	0.15	-0.12	WCC	-0.21	-0.45#
SBP	-0.12	-0.04	URATE	0.13	-0.03	PC	-0.12	-0.03
DBP	-0.01	-0.14				PRPPC	-0.19	0.06

*<0.05 #<0.01 **<0.005 ##<0.001

See Table 7.1 for abbreviations

Table 7.8. Correlations between the mean PCR and measures of platelet aggregation in the case and control groups.

	Controls	Cases		Controls	Cases
LT50ADR	0.27*	0.01	RADR	-0.22	0.16
LT50ADP	0.03	0.05	RADP	-0.20	0.03
LT50COL	0.22	0.25*	RCOL	-0.29*	-0.14
LAGCOL	0.16	0.34#			

*<0.05 #<0.01 **<0.005 ##<0.001

See Table 7.2 for abbreviations

Table 7.9. Correlations between the mean PCR and plasma fatty acids in the control and case groups.

	Controls	Cases		Controls	Cases
Myristic	-0.11	0.23	Eicosenoic	0.13	-0.02
Palmitic	0.03	0.26*	Eicosatrienoic	0.07	-0.08
Palmitoleic	0.03	-0.01	Dihomo- γ -linoleic	0.02	-0.19
Stearic	0.07	-0.14	Arachidonic	0.10	-0.03
Oleic	-0.04	-0.30*	Eicosapentaenoic	-0.03	-0.12
Linoleic	0.03	0.17	Docosapentaenoic	-0.25	-0.29*
alpha-Linolenic	0.29	0.12	Docosahexaenoic	-0.08	-0.29*

* <0.05 # <0.01 ** <0.005 ## <0.001

See Table 7.3 for abbreviations.

Table 7.10. Regression analysis with the PCR as the dependent variable and the parameters having a linear association (MPV, alcohol intake, BSL, MCV and scapular skinfold thickness, C16:0, C14:0, C18:1w9, C22:5w3 and C22:6w3) with the PCR in either group as the independent variables for each study group.

Independent Variable	B	SE B	Beta	R sq	P value
A. Control Group:					
Alcohol	0.250	0.088	0.473	0.287	0.0106
BSL	-0.735	0.276	-0.445	0.481	0.0153
B. Case Group:					
C18:1w9	-0.026	0.010	-0.494	0.244	0.0165

See Tables 7.1 and 7.3 for abbreviations

7.3 ALPHA-GRANULE RELEASE ASSESSED BY PLASMA BETATHROMBOGLOBULIN LEVEL

7.3.1 Introduction

The concept of measuring platelet specific proteins was developed as a method, it was believed, for evaluating *in vivo* platelet function. The assessment of *in vivo* platelet function by other methods such as the platelet survival time is neither specific nor sensitive enough to evaluate the relationship between platelets and vascular disease.^{94,827,828} The differences from normal in platelet survival time are very small and not always reproducible.^{530,829} *In vitro* aggregation tests are more useful for studying bleeding disorders due to defects in platelet function and for monitoring pharmacological agents, but they have not been demonstrated to be consistently useful for identifying thrombotic abnormalities.⁵³⁰ Therefore, the measurement of plasma BTG is used as a measure of *in vivo* alpha-granule release in this study.

7.3.1.1 Human platelet alpha-granule release. BTG and PF4 are released following platelet activation and degranulation. Their presence in plasma can be regarded as an *in vivo* marker of platelet activation.^{76,77,88,320-324} The major storage sites for platelet secreted proteins are the alpha-granules.⁸³⁰ During the platelet release reaction the contents of platelet granules are liberated, and this may occur without platelet aggregation.⁸³⁰ The proteins released, which include BTG, PF4 and fibrinogen, can be identified morphologically in platelets and are the main constituents of a subcellular fraction of lysed platelets.^{311,830} Alpha-granules appear to be vesicles sealed by a membrane that possesses GP IIb and GP IIIa receptors which are the same as those on the whole platelet membranes. The proteins are excreted by exocytosis from activated platelets.^{311,337,830} These proteins are broadly divided into two groups, those homologous with other plasma proteins, and those proteins specific for the platelet.⁸³⁰ Proteins secreted by the platelet, but also found in the plasma, include fibrinogen, fibronectin, albumin, Factor V, plasminogen, high molecular weight kininogen, von Willebrand Factor, as well as a number of others.⁸³⁰ Platelet specific proteins include PF4, BTG, platelet derived growth factor (PDGF), and thrombospondin.^{311,337,830}

The plasma BTG level has a wide "normal" range of reported values varying from 5 to 65 ng/ml.^{76,312,323,629} The average platelet content is approximately 18mg/10⁹ platelets.³¹² BTG has been reported to be present in increased concentrations in the plasma of patients with various vascular disorders.^{76,77,321,323} Interest in alpha-granule proteins is partly related to their proposed roles in normal and perhaps abnormal haemostasis,³³⁷ and in tissue repair following their localised release by activated platelets.⁸³⁰ The measurement of BTG may also be helpful in delineating alpha-granule deficiency states.⁸³¹ BTG is metabolised by the kidney, and therefore increased levels are found in patients with renal insufficiency,⁵³⁰ and the plasma level correlates with creatinine in these patients.^{832,833} The biological effect of BTG has been thought to involve an inhibition of PGI₂ production by endothelial cells,⁸³⁴ although this is of doubtful significance.^{530,830}

7.3.2 Why measure plasma BTG levels?

Platelets appear to be important in both the genesis and the subsequent thrombotic complications of atherosclerotic vascular disease.^{4,25,115,238,242,299,835} Therefore, numerous methodologies have been employed in attempts to detect platelet activation *in vivo* that may allow identification of patients at risk of developing CAD or its thrombotic complications. In this respect, BTG has been extensively evaluated. Variation in the plasma levels of BTG can be detected by relatively sensitive RIA methods.^{629,836-839} Since BTG is liberated during the sampling process and the preparation of PPP, methods to limit this problem have also been developed.^{833,839} Initial observations indicated that plasma BTG concentrations are elevated in states associated with intravascular platelet aggregation and release,⁸³⁸ as well as in clinical conditions with organ damage.⁶²⁹

7.3.2.1 Clinical utility of plasma BTG levels. Increased levels of BTG have been found in a wide variety of clinical conditions.^{299,830,840} Such states include venous thrombosis, CAD, AMI, cerebrovascular disease, diabetes, hyperlipidaemia, renal failure, the nephrotic syndrome, thrombotic thrombocytopenic purpura, adrenergic stimulation by cold, disseminated intravascular coagulation and cancer. In addition, plasma BTG is also increased in patients with prosthetic heart valves and extracorporeal circulation. However, measuring plasma BTG in any of these conditions has little or no practical clinical utility at present.

7.3.2.2 BTG and atherosclerotic vascular disease. Following the initial hopes that measurement of plasma BTG may provide a clinically useful tool for predicting and/or assessing thrombotic disorders,^{629,838} a series of subsequent investigations have failed to demonstrate any value for this measurement in clinical situations.^{76-83,841-843} Not that all of these studies reported negative findings. Indeed, a number demonstrated a positive association with various clinical manifestations of vascular disease.^{76,77,79,82,83,323,842,843} Elevated levels were associated with transient cerebral ischaemic episodes and partially predictive of prognosis.⁸⁴² Increased levels have also been found in stable CHD^{77,82,83} and in acute AMI.^{76,77,79} Others have interpreted the increased levels of BTG after AMI as being due to platelet interaction with previously infarcted myocardium rather than atherosclerotic coronary arteries.⁸⁴¹

Patients with documented CHD and stable angina may have a small but statistically significant increase in BTG compared with normal controls.³²³ Others have found no change in the levels of BTG in patients with chest pain, AMI or unstable angina,⁷⁸ in patients with CHD and no previous AMI,⁸⁴¹ in patients with stable CHD compared to those with coronary occlusion or acute myocardial ischaemia,^{78,82} nor in patients after exercise induced myocardial ischaemia.²⁹⁸ In addition, measurement of BTG in patients does not detect platelet involvement in acute coronary occlusion.⁸² Patients with stable CHD and the shortest platelet survival time, tend to have higher concentrations of BTG and PF4, with a significant correlation between the platelet survival measurement and plasma concentrations of these proteins.³²¹ However, the correlation coefficient is low and the overlap with normals is large.³²¹ Collectively, these studies attest to the doubtful clinical utility of this measurement.

7.3.2.3 BTG values in normals. Besides the controversy regarding the actual

value in the various vascular disease states, the lack of clinical utility arises from the large overlap between normal and abnormal population groups.^{76,530,830} This results in very poor specificity and sensitivity for predicting disease in those studies demonstrating a relationship with atherosclerotic vascular disease. BTG levels in normals range between 5 and 65 ng/ml, accounting for 95% of observed values for normals.^{76,323,629} Reported normal values do vary considerably. For example, the BTG level in normals (in ng/ml) has been reported as 24.0 \pm 1.6,⁸⁴⁴ 17.8 median (with 90% of values occurring in the range 6.6-47.9),⁵³⁰ 30.7 \pm 13.7,⁸³⁶ 28.0 \pm 18,⁸⁴⁵ and 6.0 \pm 3.6.³²³

BTG levels are influenced by a number of factors. The BTG level appears to increase with age,^{629,630,846} although this is not a uniform observation.³²³ It is possible that the age relationship only applies to the very old⁶²⁹ and not to younger age groups.⁸⁴⁵ No correlation has been found between BTG and the whole blood platelet count.^{323,629} However, the plasma BTG level is proportional to the platelet count in some conditions with myeloproliferative abnormalities,⁶²⁹ or when the platelet count is high.³²³ Little temporal variation in the plasma BTG concentration in individual subjects has been documented.⁶²⁹ Those studies evaluating BTG levels in patients with atherosclerotic vascular disease show substantial overlapping with levels found in normal subjects.^{76,323,530,830} Nonetheless, despite such a wide variation, low normal values of BTG may provide good evidence that increased *in vivo* platelet activation is not occurring.⁵³⁰ High plasma levels of BTG can be equated with increased *in vivo* platelet release only if *in vitro* release can be excluded and if the creatinine is normal.⁵³⁰

7.3.2.4 Possible explanations for the variability of results. The reported values of BTG in normals and cases show significant variability between laboratories.⁸⁴⁰ One reason for the discrepancy between results stems from differences in techniques employed in blood collection and preparation of PPP.⁸⁴⁰ Although age differences do not appear to be significant in studies of younger individuals (<50 years),^{629,845} this may not be the case in studies of older population groups.^{629,846} The significant age differences between case and control groups in a number of studies may have resulted in a biased increase in levels for the case groups, who were older than the control subjects.^{77,79,82,841}

Another possible explanation for the variability may relate to the differences in medications used between cases and controls, as well as between the population groups in the different studies. Cardiovascular drugs have the theoretical potential of reducing platelet reactivity *in vivo*.^{491,507,510,512,513,516,517,519-525,847} However, no effect on plasma BTG levels with a combination of a beta-blocker and a calcium channel blocker in a group with stable CHD, either in the presence or absence of exertional ischaemia has been demonstrated (see Chapter 4 and reference²⁹⁸).

The population groups in many previous studies tended also to be heterogeneous in other ways, having mixed gender groups,^{77,78,79,82,83,841,846} non-angiographically confirmed diagnosis of CAD without other objective documentation of disease in some of the groups studied,^{79,82} and, since the degree of left ventricular dysfunction may affect BTG levels independent of CAD,⁸⁴¹ a measure of the left ventricular function has not been reported in the majority of studies. These potential areas of bias are

directly accounted for in this study.

7.3.2.5 BTG and acute thrombotic complications of CAD. Platelets are involved in the acute occlusive ischaemic syndromes of CHD, namely, AMI^{59,91,308,767,807,848-853} unstable angina^{27,45,116} and sudden cardiac ischaemic death.^{15,27,58,238,767,584} These observations provide supportive evidence for those studies demonstrating an increase in BTG levels associated with these conditions.^{76,77,79} Indeed, marked increases in plasma levels of BTG and depletion of these proteins from circulating platelets occurs in association with acute *in vivo* platelet activation and release.³²³ There is no evidence of platelet activation being present in patients with stable CHD or non-clinical CAD.^{45,298} Nonetheless, the possibility still exists that individuals with premature CHD possess inactivated platelets but with the potential to manifest hyper-reactive function given the appropriate circumstance.²⁹⁸

7.3.2.6 Summary. The clinical studies described do demonstrate the utility of measuring BTG levels to differentiate between groups with *in vivo* platelet activation, despite the inadequacy of the measurement for diagnosis in individuals.

7.3.3 Methods

7.3.3.1 Reported methods from the literature. A standard method was originally developed to measure the plasma concentration of BTG in large numbers of samples using a RIA technique.⁸³⁹ The RIA method was more specific and sensitive than alternative immunological methods.⁸³⁹ The specific details of the method described included:

% binding of tracer in :-

zero standards	49.2 +/- 4.8
non-specific control	5.7 +/- 0.8
Detection limit (pg/ml)	714 +/- 216
Inter-assay CV %	14.5 %
Intra-assay CV %	13.7 %

The antibody to ligand binding required up to 24 hours incubation to achieve equilibrium at 4°C.^{839,845} A subsequent variation in the methodology allowed quicker processing by reducing the incubation period to approximately 60 minutes.⁸³⁹

7.3.3.2 Methodological limitations for the measurement of BTG. Stimulation of the release reaction and subsequent *in vitro* release of BTG can easily occur during the collection and processing of PPP prior to measuring the BTG level.⁸³³ Temperature variations, physical trauma and a variety of chemicals all have the capacity to promote the release reaction of platelets.⁸⁴⁰

In the absence of other forms of provocation, the release reaction does not appear to occur at 4°C.⁸³³ Blood for estimation of *in vivo* BTG levels should be directly collected into a mixture of EDTA and platelet inhibitors, such as prostaglandin E1 (PGE1) and theophylline, and maintained at a temperature of 0-4°C immediately after collection and during the processing.⁸³³

In addition, the plasma BTG level appears to be sensitive to the venepuncture technique.⁸⁴⁴ Even though the potential for BTG release exists when withdrawing whole blood through a tube inserted into a vessel,⁸⁵⁵ venous sampling undertaken appropriately and directly via a venepuncture does not influence the plasma BTG levels greatly.^{833,855} EDTA is required to prevent the formation of thrombin, a potent aggregator of platelets, during the collection.

EDTA can have an adverse effect on platelets without the modifying influence of PGE1, a low temperature and rapid processing.⁸⁵⁶ Moreover, the effectiveness of these modifying factors when platelets are *a priori* stimulated or processed poorly is controversial.⁸⁵⁶

Centrifuging blood at 1900g, which is recommended, yields PPP rather than platelet free plasma. This can result in contamination of the sample with platelets following centrifugation, ensuring spuriously high levels of BTG if these platelets are activated or lysed following freezing and thawing.⁸⁵⁷ Recommendations have therefore been made to assay the plasma before freezing in order to prevent contamination from the lysis of residual platelets following freezing, or alternatively, using high speed centrifugation to produce platelet free plasma before freezing.⁸⁵⁷ However, sampling from the middle layer of plasma may reduce the problem of contaminating platelets in the assay sample.⁸⁵⁸

7.3.3.3 Techniques to diminish methodological limitations. The blood sample should be collected immediately into a mixture of an anticoagulant (EDTA) and a platelet inhibitor (such as PGE1 and theophylline) at 4°C and maintained at this temperature during the processing, including centrifugation at 1900g for 60 minutes. This method has been demonstrated to give the lowest BTG plasma concentration for any given sample.⁸³³ The low temperature reduced the release of BTG more than the other factors evaluated.⁸³³

BTG appears not to be liberated to any extent with passage through a venepuncture device, even when forced through a small 23 gauge needle.⁸³³ However, it is released after the blood enters the syringe, and this becomes significant within 7 minutes without the appropriate anticoagulant, anti-platelet agents and the appropriate temperature described above.⁸³³ Optimal conditions exist when the blood is collected directly into a precooled syringe containing EDTA and PGE1, placed on ice immediately and quickly centrifuged at 4°C.

The PPP samples were stored at -70°C and analysed within 6 months. Furthermore, blood from controls and patients were collected in a similar time frame and analysed simultaneously. Utilisation of PPP following freezing and storing the samples, rather than immediate analysis did not appear to have a major impact on the BTG values. In addition, the middle-layer plasma, recommended as having the least contamination of residual platelets after centrifugation,⁸⁵⁸ was always used. Despite all the precautions described above, false positive results do occur because of unsatisfactory venepuncture and preparation of PPP.⁸⁴⁰

7.3.3.4 Biological variation. The concentration of plasma BTG possibly increases with age in normals.^{629,846} Although the mean plasma BTG value for each 20 year

cohort increased slightly, the difference only becomes significant for the over 70 year old compared to the young group.⁶²⁹ In younger normal males and females (less than 45 years of age), the BTG level does not vary significantly.⁸⁴⁵ No variation in the BTG level taken over a period of 15 minutes from the one sampling site has been observed.⁸⁴⁵ There is little variation in the level of samples taken weekly or monthly over a 3 month period.⁸⁴⁵

7.3.3.5 Study Method Employed. An RIA kit (Amersham) to measure BTG levels in PPP was used and the method is described in Chapter 2. The protocol used is a modification of a previously described method.^{833,839} In particular, the modification used involves the utilisation of a more rapid method for determining the BTG level by RIA which involves:

1. incubation of the antibody-ligand mixture and hence reaction, for 60 minutes at room temperature; and
2. following incubation, ammonium sulphate was added to the solution prior to centrifugation in order to obtain a precipitate, the antibody bound I¹²⁵ labeled BTG being attached and precipitated by the ammonium sulphate solution.

7.3.4 Results

7.3.4.1 Group comparisons. Differences in the mean plasma BTG levels (\pm SD) between the control group (40.3 \pm 17.3 ng/ml) and the case group (47.0 \pm 36.9 ng/ml) was not significant. The case group had a median of 36, skewness 2.4, kurtosis 5.8, a minimum value of 15ng/ml, a maximum value of 190 ng/ml, and 9 of 53 samples with values above 50ng/ml. The control group had a median of 41, skewness 0.3, kurtosis -0.8, a minimum value of 13ng/ml, a maximum value 76ng/ml, and 12 of 48 samples were above 50ng/ml.

7.3.4.2 Correlations with CHD risk factors. Differences were found in the relationships between the various major risk factors and the plasma BTG levels in the control group compared to the case group. In the control group, the BTG level positively correlated with systolic and diastolic BP, weight, BMI and cholesterol level, while having a negative correlation with HDL cholesterol and apoprotein A values (Table 7.11). The BTG level in the case group only had a borderline positive association with systolic and diastolic BP, and possibly a positive association with age (Table 7.11).

7.3.4.3 Correlation with platelet function tests. Different correlations were also found between the plasma BTG level and platelet function tests in the two groups. In the control group, no relationship was found between BTG levels and the PCR, MPV or the PRP platelet count. The BTG level positively correlated with the whole blood platelet count and negatively correlated with the serum TXB2 level in the control group (Table 7.11). In the case group, the BTG level was only positively correlated with the whole blood platelet count and the PRPPC but not the serum TXB2 level (Table 7.11). There was no relationship between BTG levels and other blood cell indices in either group (Table 7.11).

In the control group, the BTG level was lower when *in vitro* platelet aggregation was

greater. In other words, there was a negative correlation with the rates of aggregation for collagen and adrenaline, and a positive correlation with the time to 50% aggregation for collagen and adrenaline, and collagen lag time (Table 7.12). In the case group, similar correlations were observed, but only with adrenaline as the agonist for aggregation (Table 7.12).

7.3.4.4. Correlation with plasma FA content. The relationship between the plasma BTG levels and the content of plasma fatty acids was positive with eicosenoic (C20:1n9) and stearic (C18:0) acids, and negative with palmitic acid (C14:0) in the control group (Table 7.13). This result differed from that in the case group, where a negative association with dihomo- γ -linoleic (C20:3n6) and eicosapentanoic (C20:5n3) acids, and positive association with myristic (C14:0) and palmitic (C16:0) acids existed (Table 7.13).

7.3.4.5 Regression analysis. When the plasma BTG level was considered a dependent variable in a regression model, and factors that could influence the plasma level, as determined by a linear relationship, were entered as independent variables, the plasma content of stearic (C18:0) and myristic (C14:0) acids, as well as diastolic BP, were independently predictive of the BTG level in the control group (Table 7.14). Myristic acid and diastolic BP related inversely, and stearic acid related directly to the plasma BTG level. Age, blood lipids and platelet count were not related (Table 7.14). In the case group, the age of the patient inversely, and myristic acid directly predicted plasma BTG levels; BP, blood lipids, and platelet count were not related (Table 7.14).

There is a known relationship in CHD patients between BP, glucose intolerance, specific lipoproteins, body fat distribution, and possibly plasma fatty acids. The association between the plasma BTG level and weight and BP in the control group, and diastolic BP in the case group may represent an indirect relationship with other factors not entered into the regression model. Therefore the fasting blood glucose level and HbA1C were entered as independent variables into the regression model. In addition, in view of the relationship with *in vitro* aggregation and the possibility of *ex vivo* platelet stimulation during the collection and processing of samples, plasma fibrinogen was also entered into the model. The outcome was that stearic acid maintained an independent direct predictive relationship with the plasma BTG level in the control group (Table 9). The plasma myristic acid content maintained a direct, and age an independent indirect relationship with the plasma BTG level in the case group (Table 10).

7.3.4.6 Coronary angiography. Of particular importance, the BTG level did not correlate with any of the angiographic measures of CAD in the case group.

7.3.5 Discussion

Using exactly the same methods of collection, processing and measurement of BTG, the two study groups had similar mean values. Moreover, the values obtained in the study were in the same range of normal as that reported in the literature using similar methodology.^{76,312,629} The range for both groups was well below the reported increased levels of BTG (viz 91.4ng/ml) which occur following freezing at -26°C.⁸⁵⁷

Given the limitations with the current study, and the probable contribution of an indeterminate quantity of BTG released *ex vivo* during the processing, our observations do not support the presence of continuous activation of platelets *in vivo*, as measured by plasma BTG level, in males with premature CHD compared to age matched controls without CHD.

A number of observations made in this study in the normal (control) population group are consistent with previous reports in the literature. No relationship exists between plasma BTG levels and age in younger populations of normal subjects.^{323,629,845} Furthermore, there was no relationship between plasma BTG levels and the PCR in either the case or control groups.^{79,844} A weak relationship between the level of plasma BTG and some of the plasma lipoproteins (positive with total cholesterol; negative with HDL cholesterol and apoprotein A1 levels) is consistent with the general conclusion that there is an increased reactivity of platelets in the presence of lipoprotein abnormalities.^{65,66,419,423-425,646,627,859-861} Similarly, an association with plasma fatty acids may be of relevance given the close relationship between fatty acids and platelet reactivity.^{66,627,646,862,863} The plasma content of myristic and stearic acids were independently predictive of the plasma BTG level with multivariate analysis, but the major plasma lipoproteins were not. Moreover, the different relationship between the plasma BTG level and the plasma fatty acids in the control and case groups suggests that there is a difference in platelet response between the two groups following exposure to plasma fatty acids (see Appendix 2).

An association between plasma BTG levels and the circulating platelet count was found in controls as well as patients, although the relationship was weak in the control group. In the few studies in which it was examined, no relationship was noted between plasma BTG levels and the circulating platelet count in control subjects with normal platelet counts,^{323,629} although this is not a universal observation.⁸⁶⁴ Most clinical studies have not commented on the relationship. A positive association with the whole blood platelet count would be expected if the measured BTG was in fact released following the collection and processing of the sample. For the same reason a relationship exists between the platelet count and serum BTG.³²³

The major variations between the mean values for plasma BTG levels in normal subjects, and the wide range reported in the literature, implies differences related to either conditions of measurement or possibly heterogeneity of the "normals" studied. A range of so called "normal" values for plasma BTG between 6ng/ml³²³ to 31ng/ml⁶²⁹ in well conducted studies remains unexplained, apart from the likelihood of contamination from *ex vivo* and/or *in vitro* release. Since *in vivo* alpha-granule release can occur readily without dense granule release or reduced platelet viability,^{865,866} it has been stated that the appearance of BTG in plasma provides sensitive and objective evidence of platelet activation *in vivo*.³²³ However, the observation that untransfused aplastic anaemic patients and idiopathic thrombocytopenic patients had plasma BTG levels similar to normals examined by the same methods,³²³ suggests that *in vitro* artifacts may be contributing to the BTG levels measured in plasma.

A number of other observations indicate that *ex vivo* release of BTG has occurred.

The inverse correlation between *in vitro* measures of platelet aggregation (viz the higher the plasma BTG level the less the aggregation response) for blood taken at the same venepuncture for both analyses points to the possibility of some degree of platelet activation occurring prior to the analysis. The platelet release could occur within the circulation or during the collection and processing. Following such activation, wherever the location, the reactivity of platelets to further stimulation may be reduced. This could result in a reduction in aggregation as indicated by the *in vitro* measures. Nonetheless, the study does not evaluate the exact timing of the release of BTG, whether in the circulation prior to collection, or subsequently *ex vivo* and/or *in vitro*. To more accurately evaluate this possibility, at a minimum it would be necessary to measure simultaneously PF4,^{323,530,830} urinary excretion of the metabolites^{323,830} and the content of the alpha-granules before and after collection.^{323,830} If it is assumed that some, or possibly all, of the BTG measured was formed during processing, then plasma BTG levels may be considered a de facto measure of how efficient the collection and processing of the plasma samples was in preventing *ex vivo* platelet alpha-granule release or activation.

Blood taken sequentially from the same venepuncture, to measure plasma BTG and to determine TXB2 in the clotted whole blood, demonstrated an inverse relationship between plasma BTG and serum TXB2 in normals, but not in the patient group. In the normal subjects, it is conceivable that there is a variable and complimentary contribution to platelet activation by products of the release reaction and by thromboxane. In other words, the more that alpha-granule release contributes to platelet activation, the less that thromboxane is required to contribute to platelet activation. The absence of a relationship between plasma BTG level and serum TXB2 production, and the weaker association between *in vitro* platelet aggregation and the plasma BTG level in the case group, may indicate an alteration in the contribution to aggregation by released alpha-granule products and thromboxane compared to normal subjects. If BTG and other alpha-granule constituents were released prior to clotting, a reduced anti-heparin effect during subsequent clot formation, may lead to reduced thrombin formation and hence reduced TXB2 production. Although this is only speculative, it does demonstrate the importance, in fact the necessity of understanding the normal dynamic relationships between the subcellular components of clotting.

Of particular importance, no relationship between plasma BTG levels and the extent and severity of angiographically defined CAD was observed. Conflicting results have been previously observed using scoring methods and patient populations which would reduce the likelihood of determining an exact association.^{77,841}

7.3.6 Summary

This study was not designed to evaluate whether there is in fact a circulating level of alpha-granule proteins, nor whether the protein detected are a consequence of *in vitro* release. A study more specifically designed to answer such a question³²³ has reported the lowest "normal" plasma value for BTG available in the literature. However, even that study did not conclusively exclude *ex vivo* release as a cause of even the low level of plasma BTG (6 ng/ml) documented by the authors.³²³ The aim of the present study was to compare plasma levels in two groups using the same

methodology, acknowledging the probability that the levels observed will reflect in part *ex vivo* alpha-granule release. The relationship between the plasma BTG level and the circulating platelet count, the *in vitro* measures of platelet aggregation and the serum TXB2 levels, as well as the fact that the "normal" mean value was greater than the lowest previously described value, would indicate that some, if not all, of the plasma BTG measured reflects *ex vivo* release. If this was the case and the amount of BTG produced was the same in both groups, and considering that a difference was found between the case and control group for the PCR (indicating greater activation of platelets during blood collection and processing for the case group), then one inference is that the increased activation in the case group was not due to an exaggerated release response.

Despite these reservations, the results do indicate the following:

1. There is no measurable difference in the release of BTG from alpha-granules following collection and processing of plasma samples in young males with or without CHD;
2. There is no evidence that an *in vivo* process is occurring that causes significant release of alpha-granules in young males with stable CHD at a time when there is no evidence of acute thrombotic occlusive manifestations of CHD.
3. There is no support for the concept that an alteration in platelet reactivity associated with alpha-granule release is involved in the pathogenesis of atherosclerosis. However, the results do confirm the presence of a qualitative difference in the relationship between a measure of platelet function and plasma fatty acids between young males with CHD and a normal control group.
4. It is unlikely that the classic CHD risk factors exacerbate or cause platelet hyper-reactivity involving alpha-granule proteins, although plasma fatty acids may do so.

Table 7.11. Linear correlations (r value) between BTG levels and potentially associated variables in the control and case groups.

	Control	Case		Control	Case		Control	Case
DOB	0.15	0.30#	CHOL	0.25	-0.14	TXB2	-0.38*	-0.03
PACKYR	0.01	-0.13	TRIG	0.13	0.25	PRPPC	0.16	0.33*
ETOHGM	-0.03	0.10	HDL	-0.31*	-0.17	MPV	0.07	0.05
WT	0.29*	0.03	APOA	-0.34*	-0.20	MPCR	0.10	0.03
HT	0.19	0.04	APOB	-0.06	-0.17	Hb	0.04	0.11
BMI	0.26*	0.12	BSL	-0.00	0.08	Hc	0.13	0.15
TRICEP	-0.09	0.04	HbA1C	0.02	-0.00	MCV	0.12	0.16
SCAP	-0.18	-0.17	FIBR	0.03	0.18	WCC	0.06	0.21
SBP	-0.29*	0.18	URATE	-0.06	0.14	PC	0.29*	0.30*
DBP	-0.36#	0.22*						

*<0.05 #<0.01 **<0.005 ##<0.001

See Table 7.1 for abbreviations

Table 7.12. Linear correlations (r value) between BTG levels and platelet aggregation variables in the control and case group.

	Controls	Cases		Controls	Cases
LT50ADR	0.57##	0.39#	RADR	-0.31*	-0.38*
LT50ADP	-0.11	-0.04	RADP	-0.17	-0.10
LT50COL	0.41**	0.02	RCOL	-0.41**	0.09
LAGCOL	0.41**	0.07			

*<0.05 #<0.01 **<0.005 ##<0.001

See Table 7.2 for abbreviations

Table 7.13. Linear correlations (r value) between BTG levels and plasma fatty acids in the control and case groups.

	Controls	Cases		Controls	Cases
Myristic	-0.32#	0.39#	Eicosenoic	0.24*	0.10
Palmitic	-0.20	0.25*	Eicosatrienoic	-0.13	0.01
Palmitoleic	-0.04	0.07	Dihomo-y-linoleic	0.17	-0.27*
Stearic	0.31*	-0.21	Arachidonic	0.08	-0.04
Oleic	0.08	-0.14	Eicosapentaenoic	0.04	-0.28*
Linoleic	0.06	-0.14	Docosapentaenoic	-0.05	-0.17
alpha-Linolenic	-0.19	-0.12	Docosaheptaenoic	-0.01	-0.02

See Tables 7.1 and 7.3 for abbreviations

Table 7.14. Regression analysis with plasma BTG level as the dependent variable and DOB, weight, BMI, diastolic BP, systolic BP, cholesterol, HDL cholesterol, triglyceride, apoprotein A1, platelet count, and myristic, palmitic, stearic and eicosenoic acids as independent variables.

Dependent Variable	Independent Variable	B	SE B	Beta	Rsq	Sig
A. Control Group:						
BTG	C18:0	0.001	0.001	0.376	0.297	0.023
	Diastolic BP	-0.007	0.002	-0.421	0.419	0.005
	C14:0	-0.001	0.001	-0.330	0.493	0.050
B. Case Group						
BTG	C14:0	0.001	0.001	0.664	0.188	0.000
	DOB	0.027	0.007	0.515	0.372	0.000
	Weight	0.001	0.001	0.313	0.460	0.017

B=regression coefficient, Rsq=coefficient of determination.
See Tables 7.1 and 7.3 for abbreviations.

Table 7.15. Regression analysis with plasma BTG level as the dependent variable and DOB, weight, BMI, diastolic BP, systolic BP, cholesterol, HDL cholesterol, triglycerides, apoprotein A1, platelet count, and myristic, palmitic, stearic and eicosenoic acids as independent variables in the control group, as well as blood glucose level, HbA1C and fibrinogen levels.

Dependent Variable	Independent Variable	B	SE B	Beta	Rsq	Sig
A. Control Group:						
BTG	C18:0	0.001	0.0002	0.671	0.451	0.0002
B. Case Group:						
BTG	C14:0	0.001	0.0002	0.558	0.158	0.0008
	DOB	0.022	0.007	0.495	0.372	0.0024

B=regression coefficient, Rsq=coefficient of determination.
See Tables 7.1 and 7.3 for abbreviations

7.4 THROMBOXANE

7.4.1 Introduction

Thromboxane A₂ (TXA₂) is an arachidonic acid metabolite which is generated by platelets during aggregation. Arachidonic acid is metabolised by cyclooxygenase to form cyclic endoperoxide intermediates (PGG₂ and PGH₂). PGH₂ is subsequently metabolised by thromboxane synthase to TXA₂, a potent vasoconstrictor and stimulus for ongoing platelet aggregation.^{577,867} The important role of enhanced thromboxane generation in the circulation for local vasoconstriction, platelet activation and arterial thrombosis is widely acknowledged^{867,868} However, TXA₂ is cleared very rapidly from the blood stream, the half life of TXA₂ being only about 30 secs.⁸⁶⁹ Indeed, TXA₂ and eicosanoids in general are characterised by episodic production, and both metabolic and chemical instability.^{308,868} Therefore, the measurement of TXA₂ is difficult, and the use of standard RIA methods impractical.^{503,868}

Quantitative methods have been developed to measure metabolic products of TXA₂ which are present in the circulation longer and reflect the amount of TXA₂ produced. The very high chemical instability of TXA₂ results in the rapid non-enzymatic hydrolysis of TXA₂ and formation of TXB₂, the inactive hydration product of TXA₂.^{503,868} However, the measurement of TXB₂ in peripheral venous plasma does not reliably reflect changes in TXA₂ synthesis and release *in vivo*⁸⁶⁸ because TXB₂ is metabolised within 5 to 7 mins in the circulation, and artifactual release occurs as a result of even a minimal degree of platelet activation during the collection process.^{503,868} The use of *ex vivo* measurements of plasma TXB₂ as a measure of TXA₂ production *in vivo* have not been clinically useful due to methodological problems, and they are potentially misleading when utilised for research purposes.^{503,575,868}

At present there are no reliable and sensitive plasma markers specific for platelet production of "in vivo" TXA₂, which are not influenced by the collection and processing of blood samples.^{504,503,868} Circulating long-lived metabolic products of TXB₂, such as 2,3-dinor-TXB₂ and 11-dehydro-TXB₂, may circumvent the problems of *ex vivo* activation, but frequent sampling around a clinical event is required, the timing of release is uncertain, and the vascular location and the tissue origin of the TXA₂ is also uncertain.⁸⁶⁸ The measurement of stable metabolic products excreted in the urine has been more reliable in reflecting the *in vivo* production of TXA₂.^{503,868,870,871} However, similar potential limitations inherent in the measurement of these products also exist.⁸⁶⁸

The ability of platelets from individuals with atherosclerotic vascular disorders to form TXA₂, and thus TXB₂, and the degree of production is controversial.^{308,835} Doubts about the validity of many studies relating abnormalities of TXB₂ formation to atherosclerotic vascular disease have been convincingly raised.^{503,835,868,872} Immunoreactive TXB₂ has been reported to be undetectable in human plasma obtained from healthy volunteers. Methodological difficulties and also sampling artifacts are cited as reasons why many studies investigating the endogenous biosynthesis of TXB₂ may be invalid.^{308,503,868} These difficulties, and the variability of TXB₂ measurement, are illustrated by the wide range of TXB₂ levels reported, there being

large differences in levels observed between and within studies.^{498,628,630,873} Despite such potential pitfalls, a number of studies have demonstrated quantitative differences in TXB2 formation in individuals with vascular disease. Many of these were comparison studies in which the same errors would apply to the compared groups. Thus, differences in such studies may be qualitatively valid, albeit not quantitatively.

7.4.2 Thromboxane B2 and atherosclerotic vascular disease

The endogenous production of thromboxane and PGI2 appears to be greater in apparently healthy older individuals than younger ones.^{582,874} It has also been observed that atherosclerotic individuals, with evidence of *in vivo* platelet activation, produce increased amounts of thromboxane^{332,874} and PGI2.^{874,875} An understanding of eicosanoid metabolism in normal subjects and those with clinical manifestations of atherosclerosis remains incomplete.^{308,868}

7.4.2.1 Myocardial infarction and sudden ischaemic death. Evidence of platelet activation in AMI is supported by reports of platelet aggregates in the major epicardial and intra-myocardial vessels in patients with sudden cardiac death.^{25,26,854,876} Animal studies have also demonstrated circulating platelet aggregates in the coronary sinus of animals with coronary artery occlusion,⁸⁷⁷ and platelet microthrombi in arterioles and capillaries following experimental coronary occlusion.⁸⁷⁸ TXA2 is considered one, and possibly a major mediator of platelet involvement.⁸⁷⁹

An increase in the level of thromboxane has been demonstrated after coronary artery occlusion.^{83,498,853,873,880} Elevated levels of TXB2 have been found in peripheral blood⁸⁸¹ and coronary sinus blood^{44,498,882} in association with episodes of myocardial ischaemia. An elevation of TXB2 in the coronary sinus has been correlated with lactate production (a measure of myocardial ischaemia), suggesting a relationship between TXB2 formation and ischaemia.⁴⁹⁸ Using peripheral venous blood, others found higher levels in only 39% of patients with unstable angina or acute AMI and the increase was confined to those with recurrent angina at rest and not to patients who had stabilised.⁸³ TXB2 levels were not related to the extent of CAD.⁸³

7.4.2.2 Stable and unstable angina. Most studies support the likelihood of platelet activation being involved in the syndrome of unstable angina,^{44,45,873} without substantiating a causal relationship and establishing the absence of an association with stable angina.^{44,45,298} Alterations in platelet function may also have prognostic implications for patients with unstable angina.⁴⁶ There appears to be no relation with *in vivo* TXB2 production and the severity of CAD.^{44,873}

7.4.2.3 Induced myocardial ischaemia. The release of TXB2 into the coronary circulation may accompany provocation of myocardial ischaemia with rapid cardiac pacing^{498,499} and sustained isometric exertion.⁴⁹⁹ However, the increase in TXB2 only occurred in those with unstable angina or recent AMI, and not in those with either chronic stable angina or non-ischaemic pain.⁴⁹⁹ When pacing induced ischaemia is manifested by the development of angina, a rise in lactate production and/or a decrease in lactate extraction, then TXB2 levels increase in the artery and/or the coronary sinus in the majority of patients.⁴⁹⁸ Unfortunately the controls were not

clearly defined and the subjects were given parenteral contrast before the sampling. In another study, a much greater increase in arterial and venous TXB2 levels following rapid atrial pacing was found in patients with "spontaneous" angina compared to those with stable angina.⁴⁹⁷ As in most studies, a wide variation in levels was found, although most patients with unstable symptoms had abnormal plasma TXB2 concentrations.⁴⁹⁷ The majority of stable patients had levels in the normal range.⁴⁹⁷ A greater increase in TXB2 production in the arterial and coronary venous systems following atrial pacing was observed in patients with unstable symptoms compared to stable patients with ischaemia induced by pacing.⁴⁹⁷ Release of TXA2 appeared to be greater during spontaneous angina than with pacing in patients with CHD.⁴⁹⁷ Others have provided evidence that there is no evidence of platelet activation across a normal or an atherosclerotic bed at rest.⁴⁷⁶ When the coronary blood flow is increased in the presence of obstructive CAD in the epicardial arteries, platelets are activated and aggregate more easily.⁴⁷⁶

7.4.2.4 Summary. There is convincing evidence that TXB2 formation may have a role in the pathogenesis of unstable angina, although the possibility of a causative relationship remains inconclusive. Furthermore, it appears from the majority of the studies discussed, that the activation of platelets and/or the production of TXB2 across the cardiac vasculature is independent of the extent of coronary artery atherosclerosis,^{497,873,883} although platelets may be activated in the presence of severe obstructive disease and increased coronary blood flow.⁴⁷⁶

A role for platelet involvement in chronic stable angina of effort, as measured by the "in vitro" presence or production of TXB2, has not been substantiated.^{298,308} However, the methodology of using peripheral venous blood has been placed in question.^{45,499,868} Measurement of the transcardiac gradient may be more valid as peripheral plasma concentrations do not accurately reflect intracardiac concentrations, production or release.⁴⁴ The methods involved in intracardiac sampling allegedly are valid for measuring plasma TXB2.^{499,497}

Despite the methodological difficulties, in general, these studies are consistent with present clinical information which supports the role of platelet activation in unstable angina,^{97,884} a condition in which thrombus formation occurs in association with varying degrees of atherosclerotic coronary arterial narrowing.^{24,884}

7.4.3 Thromboxane and risk factors for atherosclerosis

Numerous studies show that some of the major risk factors have an adverse effect on platelets resulting in an increase in platelet reactivity. The effect of these risk factors on platelet function, and on prostaglandin and eicosanoid metabolism in particular, has attracted considerable attention.

7.4.3.1 Hyperlipoproteinaemia. Platelet membrane cholesterol content and circulating lipoproteins may lead to an altered platelet function.^{124,128,425,505,628,859,885} This is discussed in more detail in Chapter 5. Others have found that the major risk factors for CAD do not influence platelet TXB2 production.⁵⁰⁴ Moreover, it has been suggested that the capacity of platelets to form TXB2 is not altered per se by atherosclerotic vascular disorders, and that the normal homeostatic mechanism can

be triggered in response to local abnormal stimuli without requiring heightened platelet reactivity.⁵⁰⁴

7.4.3.2 Diabetes mellitus. The literature concerning thromboxane biosynthesis, platelet function and diabetes mellitus is also controversial. Reports that platelets from diabetic subjects release increased quantities of TXB₂ has been extensively reviewed.^{140,141,826} Using methodologies which are allegedly specific and sensitive to thromboxane production, without the problems of *ex vivo* platelet activation, no evidence of a primary disorder in the generation of either thromboxane or PGI₂ has been documented in insulin dependent diabetes mellitus.¹⁴¹ However, insulin dependent diabetics have an increased sensitivity to platelet aggregation by ADP, adrenalin and collagen.^{141,826} Moreover, platelets from diabetics with vascular complications show significantly greater TXB₂ synthesis from exogenous arachidonate when compared to platelets harvested from controls and diabetics with no complications.⁸²⁶

7.4.3.3 Tobacco consumption. There is some evidence to indicate that tobacco may have an adverse effect on platelet reactivity.^{118,121,122,427,430-432,886-890} An improvement in platelet survival time has been observed in smokers, with or without CAD, after a period of abstinence from tobacco.³²⁰ Smoking tobacco seems to have an immediate effect on platelet function, increasing platelet aggregability¹²² which is independent of nicotine concentration.¹²³

7.4.4 Peripheral vascular disease

The involvement of platelets in the formation and/or symptomatic presentation of peripheral vascular disease is also currently being intensely investigated. Platelets accumulate at points of arterial endothelial damage, forming microthrombi of platelet aggregates and fibrin.⁸⁹¹ It is postulated that these can become organised and incorporated into the vessel wall.⁸⁹¹ Furthermore, when platelets are exposed to arterial sub-endothelium, they are stimulated to release intracellular contents such as BTG, PF₄ and ADP, and also to synthesise TXA₂.^{830,836,840} In severe peripheral atherosclerotic vascular disease there may be an increased excretion of the metabolic products of thromboxane (such as urinary secretion of 2,3-dinor-TXB₂^{332,563,870} and 11-dehydro-TXB₂.⁸⁷⁰

Atherosclerotic individuals (subjects with peripheral vascular disease and CHD) with evidence of increased platelet activation *in vivo*, produce increased amounts of both thromboxane^{45,332,563} and PGI₂.^{45,875} The endogenous production of thromboxane and PGI₂ is greater in apparently healthy older individuals than in the young,⁵⁸² although not as markedly increased as in subjects with severe atherosclerosis.^{45,563,874,892} Furthermore, in patients with peripheral vascular disease this indirect measure of *in vivo* activation seems to persist for some weeks.^{582,892} New platelets which are formed and which have increased reactivity suggest chronic platelet activation is occurring. Indeed a subgroup of patients with severe peripheral vascular disease have elevated BTG levels, circulating platelet aggregates, shorter bleeding times and increased secretion of the 2,3-dinor- and 2,3,-dinor-6 keto metabolic products of TXA₂ and PGI₂ respectively.³³² Some of the patients in this study also had CHD, some had insulin dependent diabetes and some were still

smoking. Therefore, the cause of the platelet activation remains unknown. Despite the presence of these confounding factors, evidence of a chronic state of activation does exist,⁸⁹³ but the cause of such a state is uncertain.

These findings provide indirect evidence of platelet activation *in vivo* in such patients with angiographically confirmed severe peripheral vascular disease, having rest pain with or without ischaemic foot ulceration. However, it is not clear whether the atherosclerosis itself caused platelet activation, or whether ischaemia caused the platelet activation, and whether increased platelet reactivity aggravated the peripheral ischaemia. Nevertheless, the finding would indicate that the pathophysiologic mechanisms causing rest pain in peripheral vascular disease are similar to those occurring in CHD. The question of a cause or effect association remains unresolved.

7.4.5 Conclusions

Increased platelet eicosanoid metabolism is associated with acute obstructive ischaemic coronary artery syndromes. In addition, increased platelet eicosanoid metabolism may be chronically associated with severe peripheral vascular disease.⁸⁷⁴ A persistent increase in thromboxane production does not occur in stable CHD. Persuasive evidence exists that abnormal cholesterol metabolism is associated with potentially increased thromboxane production. However, the relationship, if any, between TXB₂ formation and the other major risk factors for atherosclerosis remains to be clarified. If platelets have any role in the pathogenesis of coronary atherosclerosis, as has been frequently postulated,²⁴² it is possible that risk factors may impact on the early subclinical formation of coronary atheroma by enhancing platelet activation.

Currently, there is equivocal support for the notion that platelets from patients with CHD have the potential for increased reactivity, given the appropriate stimuli. Such possible stimuli include the major CHD risk factors. Further research to determine if increased reactivity of platelets is unequivocally present in patients with CHD is required. In particular, there is a need to further clarify the relationship between thromboxane production and stable CHD.

In this study, the relationship between plasma and serum TXB₂ levels and the major risk factors in a homogeneous group of young male patients with CHD, and a homogeneous control group, is examined. In addition, the relationship of these measures to angiographically defined CAD in the patient group has been explored (see Chapter 8).

7.4.6 Methods

The methods employed to measure TXB₂ are described in Chapter 3, Chapter 8 and Appendix 1.

7.4.7 Results

7.4.7.1 Group differences. As was found in one of our preliminary studies,²⁹⁸ the majority of the plasma TXB₂ levels were below the sensitivity of our RIA (*viz.*

40 pg per ml). Both groups had less than 10 individuals with any detectable plasma TXB₂, and this was assumed to be related to *ex vivo* release. Therefore, the assessment of plasma TXB₂ in relation to other parameters was not undertaken.

The case group had a significantly higher serum TXB₂ level than the control group, although the values in both groups had a broad standard deviation and extensive overlap (group means \pm SE were 111 \pm 12 ng/ml for the case group vs 61 \pm 9 for the control group, $p=0.0004$).

7.4.7.2 Correlation with major risk factors for CHD. Major qualitative differences existed between the two groups for the linear relationships between serum TXB₂ and risk factors for CHD (Table 7.16). In particular, in the normal group there was a positive relationship between serum TXB₂ and blood glucose level and diastolic BP. In the case group, there was a positive relationship between serum TXB₂ and prior tobacco consumption, BMI and the plasma fibrinogen level (Table 7.16).

7.4.7.3 Correlation with blood cell indices. The case group had significant associations between serum TXB₂ levels and other blood cell indices (Hb, Hc and WCC) which were not present in the control group (Table 7.16).

7.4.7.4 Correlation with plasma fatty acids. The control group had consistent and qualitatively different relationships between the plasma fatty acid content and the amount of TXB₂ produced compared to the cases (Table 7.17). Palmitic, palmitoleic and eicosenoic acids correlated inversely and eicosadienoic, dihomo- γ -linoleic, arachidonic, eicosapentaenoic, docosohexanoic and docosopentaenoic acids correlated directly with the amount of serum TXB₂ in the control group only (Table 7.17).

7.4.7.5 Correlation with other measures of platelet function. In the control group serum TXB₂ levels were associated negatively with plasma BTG levels and the PCR. In comparison, in the case group serum TXB₂ levels were associated negatively with the MPV and positively with the platelet count (Table 7.16). In the control group there was a stronger association between serum TXB₂ levels and *in vitro* measures of platelet reactivity (platelet aggregation and responses to various agonists) than in the case group (Table 7.18).

7.4.7.6 Regression analysis with serum TXB₂ as the dependent variable. All those parameters which had a linear correlation with serum TXB₂ and which could possibly influence the formation of TXB₂ were entered into a stepwise multivariate regression model for both groups. This was carried out to determine which variables, of those evaluated, independently predicted the level of TXB₂ produced during clotting of whole blood. In the control group, only some of the fatty acids (eicosatrienoic, eicosenoic and eicosopentaenoic acids) and the blood glucose level maintained an independent association (Table 7.19). In the case group only the MPV maintained a predictive relationship with serum TXB₂ levels (Table 7.19). These results indicate qualitative differences between the two groups with regard to mediators of *ex vivo* thromboxane production.

The observations in this case-control study are consistent with previous reports showing no increased thromboxane production *in vivo* in patients with stable CHD.^{45,298,503} However, clear differences exist between the two groups with regard to production of thromboxane during *in vitro* clotting of whole blood. This increased *in vitro* production is induced by endogenous platelet agonists, although the molecular environment which occurs during *in vivo* thrombus formation is not necessarily replicated. In addition, the potential effect on thromboxane metabolism by the major CHD risk factors may have led to the difference observed.

7.4.8.1 Risk factors and thromboxane. Both nicotine *in vitro* and smoking *in vivo* inhibit vascular PGI₂ synthesis and augment platelet reactivity.⁸⁹⁴ With *ex vivo* clotting there was no relationship between previous tobacco consumption and TXB₂ production in the control group. Similar observations concerning serum TXB₂ and smoking have previously been demonstrated in normal subjects.⁵⁰⁴ No relationship has previously been observed between plasma TXB₂ levels and smoking habits in normal subjects or patients with CHD.^{83,895} In the group of young males with CHD, a linear relationship between serum TXB₂ levels and prior tobacco exposure did exist. However, the positive association between prior tobacco intake and TXB₂ formation in clotting whole blood was not shown to be an independent predictor with multivariate analysis, and the association may be determined by other unmeasured mediators or other factors such as plasma fatty acids.

Cholesterol has been reported to enhance the conversion of arachidonic acid to TXB₂,⁸⁶⁷ and an influence of lipoprotein abnormalities on eicosanoid and prostaglandin metabolism is well documented.^{124,128,505,628,885,867,885} Furthermore, the rate of serum TXB₂ production is increased in patients with hypercholesterolaemia,⁶²⁸ a positive correlation has been observed between LDL cholesterol and apoprotein B levels, and TXB₂ formation in clotted whole blood,⁸⁸⁵ and a negative correlation has been observed between serum TXB₂ and HDL cholesterol levels.⁸⁸⁵ Nevertheless, others have found no association between major risk factors, apart from an association between hypertriglyceridaemia and plasma TXB₂ values.⁸⁹⁵ Both of our study groups were neither selected nor excluded on criteria relating to lipoprotein abnormalities. Major lipoprotein abnormalities were not present in either group, although group mean lipid values for the case group had predictable differences from the control group. Since previous cholesterol levels appear to be greater than those measured at the time of the study, the levels observed may have been confounded by dietary alteration in the case group which has resulted in a reduction in plasma lipids following the diagnosis of their disorder, although no patient was taking lipid lowering medications. Therefore, the lack of an association between lipoproteins and serum TXB₂ cannot be interpreted reliably. If an association existed previously, then the influence must be reversible by dietary modification. Despite these considerations, similar negative results have previously been observed in normals.⁵⁰⁴ Furthermore, others have found that the major risk factors for CHD have no influence of platelet TXB₂ production.^{504,896} This observation is supported by our current observations, particularly when one considers the lack of predictability in the multivariate regression analysis.

One observation of interest is the positive association between the blood glucose level and serum TXB2 levels in the control group. Previous studies have shown that platelets in diabetic subjects release increased quantities of thromboxane^{140,826} and this was thought to indicate a pathogenic relationship between enhanced platelet reactivity and diabetic complications.⁸⁹³ However, the relationship between abnormal platelet function and diabetes is controversial. Altered platelet function may not precede the development of microvascular complications in patients with insulin dependent diabetes (who lack macrovascular disease and have normal renal function).⁸⁹⁷ The evidence that severe atherosclerosis involving large vessels is associated with abnormalities of thromboxane and PGI2 biosynthesis is convincing.^{332,874,875,893,895,898,899} Therefore, alteration in platelet function in diabetics, and for that matter in persons with CHD, may result from the development of large vessel atherosclerosis.⁸⁹⁷ Our observations of a positive association between the blood glucose level and the amount of thromboxane produced in clotting whole blood in a young non-diabetic male population without clinical atherosclerosis supports those studies indicating a relationship between thromboxane production and blood glucose levels independent of the presence of atherosclerosis.^{140,826}

Controversy exists as to the importance of the relationship between serum TXB2 production and other blood cells. There was no relationship in the control group between various blood cell indices and serum TXB2. In the case group, on the other hand, there was an association between blood cell indices and serum TXB2, although not sufficiently robust and independent to continue to be predictive of the serum TXB2 in the multivariate regression analysis. The necessity for evaluating the effect of other blood cell components was validated and discussed in Chapter 4 in detail.

In the control group there was a positive relationship between serum TXB2 levels and plasma polyunsaturated fatty acids, and a negative association with palmitic acid. The addition of polyunsaturated fatty acids to a diet has been shown to alter platelet fatty acid content and reduce TXA2 production.⁸⁹⁴ In our study the patients with CHD have plasma fatty acids profiles which differ from matched controls, and the plasma fatty acids in the CHD group are associated with quantitative and qualitative differences in platelet aggregability. These differences are discussed further in Appendix 2.

7.4.9 Summary

1. Thromboxane production in clotted whole blood in the case group (with CHD) appears to be quantitatively and qualitatively different from the control group. There were different influences on TXB2 production due to various plasma constituents, suggesting that an alteration in the reactivity of the platelet eicosanoid system exists in young males with CHD. Blood cell indices appear to play an important role, as well as the plasma fibrinogen level and the degree of previous tobacco intake.

2. In the case group, the most reliable predictor of serum TXB2 levels was a platelet parameter, (viz MPV), rather than external factors in whole blood. In contrast, in the control group, serum TXB2 was most reliably predicted by factors

within the blood, external to the platelet. That is, external influences within the milieu of the clot, and in particular, specific plasma fatty acids and the blood glucose level influence TXB2 production in the control group.

3. These results support the concept that the platelets of males with premature CHD function differently to those without clinical atherosclerosis, and that this may contribute adversely to *in vivo* thrombus formation.

Table 7.16. Linear correlations with serum TXB2 in controls and cases.

Variable	Control	Case	Variable	Control	Case
DOB	0.10	-0.10	CHOL	0.03	0.06
PACKYR	-0.27	0.44 #	TRIG	0.08	0.19
ETOHGM	-0.08	-0.01	HDL	0.11	-0.08
WT	-0.01	0.21	APOA	-0.06	-0.08
HT	-0.28 *	-0.14	APOB	0.02	-0.13
BMI	0.18	0.31 *	Hb	0.18	0.29 *
TRICEP	-0.02	0.25	Hc	0.01	0.26 *
SCAP	0.05	0.02	WCC	-0.02	0.42 ##
SBP	0.09	-0.16	MCV	-0.25 *	-0.17
DBP	0.27 *	-0.01			
BSL	0.26 *	0.19	PCR	-0.28 *	-0.08
HbA1C	-0.08	0.16	MPV	0.03	-0.37 #
FIBRIN	-0.16	0.34 *	BTG	-0.38 **	-0.03
URATE	-0.13	-0.03	PC	-0.01	0.43 ##

See Tables 7.1 for abbreviations.

Table 7.17. Linear correlations between serum TXB2 and plasma fatty acids in controls and cases.

Fatty Acid	Control	Case	Fatty Acid	Control	Case
Myristic	-0.06	-0.11	Eicosenoic	-0.26*	0.03
Palmitic	-0.25*	0.04	Eicosadienoic	0.48##	0.12
Palmitoleic	-0.37#	0.33#	Dihomo-γ-linoleic	0.29*	0.08
Stearic	0.21	0.07	Arachidonic	0.36#	-0.09
Oleic	-0.03	0.16	Eicosapentaenoic	0.30*	-0.22
Linoleic	0.01	-0.01	Docosapentaenoic	0.38#	0.12
alpha-Linolenic	0.12	0.12	Docosahexaenoic	0.24*	-0.36#

See Tables 7.3 for abbreviations

*<0.05 #<0.01 **<0.005 ##<0.001

Table 7.18. Linear correlations between serum TXB2 and measures of *in vitro* platelet aggregation for controls and cases.

Variable	Control	Case	Variable	Control	Case
LT50ADR	-0.28#	-0.12	RADR	0.17	0.33*
LT50ADP	-0.18	-0.11	RADP	0.34#	0.13
LT50COL	-0.43**	-0.53##	RCOL	0.33#	0.09
LAGCOL	-0.57##				

See Tables 7.2 for abbreviations

Table 7.19. Regression analysis with serum TXB2 as the dependent variable and ALL of the parameters with a linear association as the independent variables for each study group.

Independent Variable	B	SE B	Beta	R sq	P value
A. Control Group:					
C20:2n6	0.006	0.001	0.557	0.242	0.0000
C20:1n9	-0.001	0.001	-0.322	0.353	0.0085
BSL	0.026	0.008	0.366	0.455	0.0023
C20:5n3	0.002	0.001	0.326	0.559	0.0077
B. Case Group:					
MPV	-0.021	0.007	-0.499	0.249	0.0095

See Tables 7.1 and 7.3 for abbreviations

7.5 PLATELET AGGREGATION

7.5.1 Introduction

Despite the limitations of *in vitro* aggregation measures, some recent studies have indicated that the assessment of platelet aggregability is potentially a fruitful area for further appraisal.^{59,767,900-902} In particular, increases in spontaneous⁵⁹ and induced aggregation *in vitro*^{768,901,902} are predictive of coronary events and mortality.⁷⁶⁸ Furthermore, enhanced platelet aggregability is present in the morning after assuming the upright posture with a concomitant increase in plasma levels of adrenaline and noradrenaline.⁷⁶⁷ This occurs at approximately the same time as an increased diurnal incidence of AMI.^{223,224,903} Thus, platelet aggregability may be an important predictive risk factor for CHD even when the prevalence of other known risk factors for coronary events is low.⁵⁹ Not only is platelet aggregability predictive of prognosis in low risk groups of AMI survivors,⁵⁹ but it is also predictive in general population groups.^{901,902}

Platelets in patients with myocardial ischaemia appear to show more sensitivity to aggregating agents than in normal subjects.^{537,904} An enhanced platelet responsiveness to aggregating agents has also been shown in patients both early and late after AMI^{521,537,626,848,849,904} and in patients with unstable angina.⁹⁰⁵ Indeed, even in patients with stable CHD after AMI, the *in vitro* aggregation response of platelets has been found to be enhanced in some studies,^{52,91,660,904,906} although not in all.⁹⁴ The association between *in vitro* platelet function and CHD requires further examination in view of the myriad of possible confounding factors in such a relationship. This section examines the results of the *in vitro* platelet aggregation measures for the two study groups. The findings support a growing body of literature indicating that increased *in vitro* platelet aggregation is an identifiable risk factor for CHD. In addition, the relationship between aggregation and CHD is extended by evaluating the extent and severity of angiographically defined CAD.

7.5.2 Methods

7.5.2.1 Lipoprotein measurement. The methods are explained in detail in Chapters 2 and 5.

7.5.2.2 Platelet aggregation and standardisation. These methods are described in detail in Chapter 2.

7.5.2.3 Angiographic scoring methods. The methods and validation are described in Chapter 11.

7.5.3 Results

7.5.3.1 Comparison of measures of platelet aggregation. In the case group, 3 patients did not produce any serum TXB₂, and, assuming that they had used aspirin, their results were not included in the case group's aggregation measurements. The measures used to evaluate the response of platelets to platelet aggregating agents indicate that there were significant differences between the two groups in this study.

There were significantly more individuals in the CAD group who had an aggregatory response to adrenaline (Table 7.20). There were no significant differences between the threshold concentrations of each of the agonists required to achieve maximal second phase aggregation when comparing those that aggregated and excluding the non-responders (Table 7.21). The PRPPC was slightly lower in the case group compared to the controls ($294 \times 10^3/\text{ul}$ vs $328 \times 10^3/\text{ul}$, $p=0.04$), however, differences in platelet aggregation are negligible over this range in platelet counts.³³⁶

There were significant differences between the two groups for other measures of *in vitro* platelet aggregation (Table 7.22). The CHD group had more reactive platelets after exposure, in particular, to adrenaline. The time to 50% aggregation was shorter for adrenaline and ADP in the case group, but this was only statistically significant for adrenaline. The rate of aggregation was faster with all three agonists, although again, the faster rate was significant only for adrenaline. The lag time prior to aggregation for collagen was shorter in the case group, although only of borderline significance. Despite the significant differences between the group values, the variance for both groups was large and there was a considerable overlap in values between the two groups.

An interesting result was the lower platelet count in PRP in the case group. On the other hand, the MPV in the case group was larger (contributing to a larger platelet mass), therefore probably explaining the lower PRPPC. Despite having fewer platelets available for aggregation, platelets in the CHD group aggregated more rapidly in response to adrenaline when compared to the control group.

7.5.3.2 Correlations between platelet aggregation and other platelet function tests. In the control group there was a positive correlation between LT50ADR, LT50COL, and LAGCOL values and BTG levels, and a negative correlation between RADR and RCOL, and BTG levels (Table 7.23). In other words, the more active the *in vitro* aggregation response, the lower the level of BTG in plasma. The case group had similar relationships with the measures for aggregation induced by adrenaline only, but not for collagen (Table 7.23).

Also in the control group, there was a positive correlation between the PCR and LT50ADR and LT50COL, and a negative correlation with the rate of aggregation for adrenaline and collagen (Table 7.23). That is, the lower the PCR, the shorter the time to aggregation and the faster the rate of aggregation. In the CHD group the PCR was only related positively to LT50COL and LAGCOL, indicating a diminished relationship between these parameters in the case group compared with the control group (Table 7.23).

A negative correlation between serum TXB2 levels and LT50ADR, LT50COL and LAGCOL, and a positive relation with RCOL and RADP was present in the control group. Thus, the shorter the LT50ADR, the LT50COL, the LAGCOL and the faster the aggregation responses with adrenaline and collagen, then the more TXB2 was produced in clotted whole blood (Table 7.23). The relationship was similar in the case group, but only with the measures using collagen as the agonist, and the strength of the relationship was not as strong or consistent when compared to the control group (Table 7.23).

There was no relationship in either the case or control group between the MPV and any of the *in vitro* measures of platelet aggregation (Table 7.23). The PRPPC correlated directly with the various measures of platelet aggregability in both groups (Table 7.25).

7.5.3.3 Correlations between aggregation measures and CHD risk factors.

There were significant inverse correlations between measures of increasing platelet aggregation and the plasma fibrinogen level, although the relationship was only present in the control group and not in the case group. The higher the plasma fibrinogen level the lower the measures of aggregability (Table 7.23). In the control group, there was no correlation between any of the measures of platelet aggregation and blood cholesterol, apoprotein B or triglyceride levels (Table 7.24). However, in the CHD group the blood cholesterol level correlated with RADR and RADP, and inversely with LT50ADR (Table 7.24). In both groups, HDL cholesterol and apoprotein A1 levels showed a positive correlation with measures of platelet aggregation (Table 7.24).

The diastolic BP correlated with aggregation measures, but only in the control group (Table 7.25). However, the majority in the case group were using vasoactive medications. In the control group, the Hc was inversely associated with platelet aggregation (Table 7.25). There were no consistent associations between the measures of platelet aggregation and plasma blood glucose levels, alcohol intake, systolic BP, Hb and the MCV of red cells in either group (results not shown). The WCC, in the case group only, correlated with measures of aggregation (Table 7.25).

7.5.3.4 Regression analysis. To determine which of those variables having a linear association with measures of platelet aggregation independently and better predicted the degree of aggregability, they were entered into a regression model as independent variables with the aggregation measures as dependent variables.

Predictably, the higher the PRPPC the greater the aggregability in the control group (Table 7.26). In addition, in the control group, Hc and fibrinogen levels maintained an inverse association with increasing aggregability (Table 7.26). In the case group, apoprotein A1 levels had a strong positive association with aggregation, and the WCC, serum TXB2 levels and PRPPC also were associated with aggregation (Table 7.26). The aggregation response in the two groups is clearly influenced by different factors.

7.5.3.5 Aggregation and angiographic measures of CAD. The only measure of aggregation with a consistent association with angiographic measures of CAD was a measure of aggregation induced by adrenaline, the severity of CAD being associated with the rate of adrenaline-induced aggregation (LT50ADR) (Table 7.27). There was no correlation between any of the measures of platelet aggregation in response to collagen and the angiographic scores.

7.5.4 Discussion

Aggregation tests using ADP and adrenaline were previously considered to most likely differentiate between hypersensitive and "normal" control platelets.³³⁴ In this

study, the platelets from patients with CHD have been evaluated using *in vitro* methods of platelet aggregation to determine platelet sensitivity to various aggregating agonists compared to an age-matched control group, and clearly, a difference exists. The platelets from patients with premature CHD are more susceptible to aggregation as reflected by a more rapid response to platelet agonists in PRP. This was particularly evident with the response to adrenaline, providing support for a possible mechanism of increased *in vivo* platelet aggregation previously postulated.^{70,71} Furthermore, a consistent and relatively strong relationship with angiographic measures of severity and extent of CAD has been demonstrated, supporting recent studies indicating that ADP-induced platelet aggregation may be a prognostic indicator of CHD.^{59,767,901,902}

Tests performed *in vitro* on anticoagulated blood or PRP to measure platelet aggregation in response to platelet agonists are crude measures of the complex and subtle interactions that lead to thrombus formation *in vivo*.⁹⁰⁷ Such methods of assessing platelet hyperreactivity are subject to artifacts produced by blood sampling, the anticoagulants used, centrifugation and other parameters in processing the platelets, including the aggregation methodology employed.⁵⁸ These are only a few of the numerous confounding factors and avenues of potential bias with the methodology for *in vitro* platelet aggregation. These factors not only include the experimental conditions, but also intrinsic and extrinsic factors associated with platelet hypersensitivity, dietary fats, other disease states and many others.^{58,77,311,312,331,334-337} Given such methodological limitations, recent studies have used more sensitive and specific immunochemical tests.^{58,79-80,82,116,285,480,538,908} to evaluate platelets in patients following AMI⁹² and unstable angina.^{116,538} The utility of most of these measures remains under question.

Giving due consideration to the limitations of *in vitro* aggregation testing,³³⁶ they, nevertheless do provide some important information concerning the prognosis of CHD based on this and other studies.^{59,767,901,902} Therefore, there is a clear need to have a greater understanding of the epidemiology of platelet aggregation. The Northwick Park Heart Study has provided details about the variability of *in vitro* platelet aggregation which needs to be taken into account in any clinical investigation.³³¹ Platelet aggregability increased with age and fibrinogen levels, it is less in women than men at all ages (being partly accounted for by the differences in Hb and Hc), it varies in races (white races having more aggregable platelets than black), it is less in male smokers compared with non-smokers, and it has a mild inverse relationship with alcohol intake.³³¹ However, a prospective study of a normal middle aged male population showed no relationship with age when subjects within a narrow age range were examined,⁹⁰² in agreement with the present study.

7.5.4.1 Threshold concentrations of agonists for aggregation. The concentrations of agonists required for various patterns of aggregation vary in a normal population.^{765,909} In addition, an individual may have a different response on different occasions.^{765,909} Furthermore, there are no standard normal values for platelet aggregation apart from those set by individual laboratories.⁷⁶⁵ This study confirms the large variation in the threshold concentrations required to induce aggregation in different individuals. Not surprisingly such a measure was unable to differentiate between groups, much less between individuals. However, there was a marked dif-

ference in the aggregation responses to adrenaline between the two groups. The number with no response to aggregation in the control group was significantly greater than in the case group. This observation again adds to the argument that adrenaline may be involved in platelet aggregation *in vivo* and consequently in the pathogenesis of CHD.^{71,72,910}

7.5.4.2 Collagen-induced aggregation. The effect on collagen induced-platelet aggregation of the major CHD risk factors, was examined in both groups. Not surprisingly the aggregability was related to the number of platelets present in PRP in both groups, although more so in the case group.

An increased response to collagen (as reflected by the threshold concentration causing aggregation) has been observed soon after AMI.⁵² In the same study, a subgroup of young patients with CHD, with minimal or single vessel disease, had a persisting increased response.⁵² In addition, whole blood aggregation in response to collagen has been found to be associated with a greater release of TXB2 in subjects with MI, and this abnormality persists for at least 3-4 months.⁵³⁷ In the current study a similar reduction in the threshold concentration of collagen to induce platelet aggregation was not observed. Perhaps this is related to the length of time after previous MI. Also, the threshold concentration measurements have a marked variation in the values obtained between different studies, as already discussed.^{423,424,911}

Other quantitative measures of collagen-induced aggregation in the case group in the present study suggest the presence of a mildly increased response to collagen. The lag phase was shorter and the rate of aggregation was faster in the CHD patients as a group, although the results did not attain statistical significance. One study using whole blood impedance aggregometry supports the absence of a difference in platelet aggregation between CAD subjects and no CAD subjects at rest.⁴⁷⁶ No differences in platelet aggregation in response to collagen were observed between subjects with or without CAD at rest, although activation of platelets occurred across the coronary circulation following rapid atrial pacing.⁴⁷⁶

Platelet aggregability in the control group was related to the red cell mass but not the white cell number in blood. The reverse relationship held in the CHD group, with the white cell number having a strong direct relationship with aggregability. Fibrinogen levels were unrelated to aggregation in the case group, and had an inverse relationship with aggregation in the control group. Both HDL cholesterol and apoprotein A1 levels were directly related to aggregability in the case group, with the relationship of apoprotein A1 being independently associated with some measures of aggregation using multivariate analysis. The reason for these relationships remains unclear and is discussed below. Of particular interest was the finding that the amount of TXB2 produced in serum was directly related to the aggregability of platelets caused by collagen. This result is consistent with previous observations.³³⁶

7.5.4.3 ADP-induced aggregation. Platelets can be induced to aggregate with ADP in the presence of appropriate conditions (eg temperature, pH, carbon dioxide and calcium concentration, concentration of ADP and fibrinogen.³⁴³⁻³⁴⁵) There is abundant *in vitro* and *ex vivo* evidence for a partial dependence of platelet aggregation in the haemostatic response on free ADP in blood.³⁴⁸ Many previous studies,

using *in vitro* methods, have shown increased platelet sensitivity to ADP in patients with CHD.^{191,849,851,912-914} However, in patients without active ischaemia no difference has been reported between measures of *in vivo* aggregation in CHD patients and in controls, even when the patients had more reactive platelets in response to ADP *in vitro*.⁹¹⁵ Nevertheless, an association between ADP-induced platelet aggregation and AMI, both fatal⁹⁰² and non-fatal⁹⁰¹, has provided support for the concept that non-fatal and fatal CHD cases may have similar platelet abnormalities.⁴⁷⁵

A variety of aggregation responses to ADP have been observed following AMI. These include an increased response soon after AMI,^{849,851,913} increased aggregation after an early period of hyporesponsiveness,⁹¹⁴ or no increase at all.⁵² Young female survivors of AMI appear to have an increased threshold for aggregation but no difference in the rate of primary aggregation in response to ADP.⁸⁵¹ Increased platelet aggregation in response to ADP in patients with AMI is associated with an increased level of BTG suggestive of coexisting *in vivo* platelet activation.⁹¹²

In the present study various measures of *in vitro* aggregation responses to ADP were not significantly different in patients with CHD and the controls. As with collagen, there was a relationship between the platelet number in PRP (as well as in blood) and the degree of platelet aggregability in both groups. The aggregability was also related to the amount of TXB₂ able to be produced in serum in both groups. Again, there were also unexplained relationships (in the case group) between the fibrinogen level and the red cell count which were inversely related to *in vitro* aggregation, and apoprotein A1 and HDL cholesterol levels which were directly related to platelet aggregation. The amount of cholesterol in serum was also directly related to aggregability to ADP in the case group.

The lack of a difference in ADP-induced platelet aggregation in subjects with and without CHD at rest has recently been reported using different methodology,⁴⁷⁶ results which are supportive of the observations in this study. However, platelet activation was greater in the CAD subjects when provoked by rapid atrial pacing.⁴⁷⁶

7.5.4.4 Adrenaline-induced aggregation. An observation of long-standing has been that adrenaline in moderate concentrations promotes thrombosis.⁹¹⁶ Adrenaline potentiates the platelet aggregation response to other agonists.^{70-72,917} Indeed, adrenaline is considered by some to not be a primary aggregating agent but a potentiating agent for the primary agonists.⁹¹⁸ Most agonists which induce secondary aggregation only do so at concentrations much greater than physiological levels.⁹¹⁹ However, the response to low concentrations of one agonist can be potentiated by prior exposure of platelets to a similarly low concentrations of another agonist.^{910,919} Subthreshold aggregatory concentration of ADP or collagen, which are in themselves insufficient to cause platelet aggregation, may do so with the addition of adrenaline.³¹² Indeed, adrenaline potentiates aggregation induced by a number of agonists^{70-72,352,353,337} The proaggregatory effect of adrenaline probably reflects the true physiological effect of adrenaline on human platelets.⁹⁰⁷ There is a concomitant increase in alpha 2-adrenoreceptor affinity for adrenaline occurring at the same time as the increase *in vitro* platelet sensitivity to adrenaline.⁹²⁰ It may be that an enhanced affinity of platelet alpha 2-receptors to adrenaline may be one mechanism by which platelets develop hypersensitivity associated with myocardial ischaemia.⁹⁰⁵ In patients with unstable

angina there may be an increase in platelet alpha 2-receptor affinity for adrenaline⁹⁰⁵ which coincides with increased platelet aggregation and TXB2 production in response to adrenaline.⁹⁰⁵ During the acute phase of myocardial ischaemia, platelet sensitivity to adrenaline and platelet TXA2 generation is increased. Again, this parallels the increase in receptor affinity for adrenaline.⁹⁰⁵

In this study we observed an increased rate of aggregation in the CHD group in response to the adrenaline, and a significantly shorter average time to 50% change in light transmission during platelet aggregation. Both these observations indicate a greater sensitivity of platelets to adrenaline in the CHD group, even in stable patients without active ischaemia. There is considerable variability in the responsiveness of platelets to adrenaline in apparently normal subjects.^{354,355,907} Some individuals have no bleeding tendency but have platelets with a markedly reduced aggregatory response to adrenaline,^{355,907} and the second phase of the aggregatory response to adrenaline may be absent in normal subjects.⁹²¹ In the present study population, the platelets from 31% of normals and 8% of patients with CHD did not aggregate in response to adrenaline. The fact that there are significantly fewer individuals who have no aggregatory response to adrenaline at any one time in the CHD group, supports the concept of a persisting and increased sensitivity to adrenaline even during stable periods in the natural history of CHD. The absence of a response to adrenaline may reflect that it is not a primary aggregating agent. Adrenaline potentiates the platelet aggregation response for other agonists, and this proaggregatory effect may be its physiological role in platelet aggregation.⁷⁰⁻⁷²

In the present study, in both groups, the amount of TXB2 formed in serum and the blood platelet count were directly related to aggregation measures. However, the fibrinogen level and red cell mass were related to adrenaline induced aggregation only in the control group, which is consistent with previous observations.^{350,918} Once again the relationship with HDL cholesterol and apoprotein A1 levels was direct and significant, although unexplained other than possibilities such as a substitute risk factor bias. The serum cholesterol level had a consistent relationship with aggregability in the case group but not in the control group, as did the relationship with the amount of previous cigarette intake. These aspects are discussed below.

7.5.4.5 Aggregation and other measures of platelet function. The results have shown that measures of platelet function which involve *ex vivo* aggregation have a direct relationship with *in vitro* aggregability of platelets. More reactive platelets, as measured by platelet aggregation, produced a greater amount of TXB2 after maximum provocation in clotted whole blood. This observation demonstrates that the capability of platelets to produce TXB2 in serum correlates with aggregation of platelets *in vitro*. Furthermore, stimulation of platelets by collagen correlated with the reactivity of platelets as measured by the PCR. These relationships were weaker in the case group than in the control group. In contrast, the plasma BTG level was inversely related to *in vitro* aggregation responses. The higher level of circulating BTG in association with less active platelets may be attributable to prior activation. As a result of prior activation, platelets are less reactive on subsequent *in vitro* challenge.

Possible explanations for differences between the two study groups in the correla-

tions found between *in vitro* aggregation measures and the other parameters of platelet function may relate to the differences in the MPV and the PRPPC. The lower PRPPC in the case group may indicate the loss of platelets in the form of platelet aggregates during centrifugation, or, alternatively, the loss of more platelets during centrifugation due to the slightly higher MPV.

7.5.4.6 Aggregation and CHD risk factors. A relationship between increased platelet aggregability and abnormal lipoproteins has been well established in the past.^{65,66,126,421,423,424,626,627} In a population of healthy males, increased total and LDL cholesterol concentrations have been shown to be associated with increased sensitivity of platelets to adrenaline and to a lesser extent ADP.⁴²³ Hypercholesterolaemic individuals generally have enhanced *in vitro* platelet reactivity compared to normal subjects.^{424,425} There is increased sensitivity to ADP, and in particular adrenaline, in these subjects.^{424,426} In normal population groups, however, there is no relationship between cholesterol, HDL cholesterol,⁹⁰¹ or triglyceride levels,⁹⁰² and *in vitro* platelet aggregation. Similar results were observed in the present control group, in which there was no correlation between any measures of platelet aggregation and blood cholesterol, apoprotein B and triglyceride levels. In the CHD group, however, cholesterol levels did correlate with the rate of aggregation caused by adrenaline and ADP. It appears that increased *in vitro* reactivity of platelets to specific aggregating agents exists in male subjects with premature CHD which has some relationship to cholesterol levels.

There may also be a relationship between BP and platelet aggregation.¹⁴⁵⁻¹⁴⁷ In the patient and control populations of this study, the BP was well controlled at the time of blood collection and it was the same in both groups. Therefore the relationship between BP and platelets could not be adequately evaluated. Nevertheless, there was a positive linear association between diastolic BP and aggregability of platelets in response to all three agonists in the control group, an observation consistent with that found in the literature.¹⁴⁵⁻¹⁴⁷

Raised levels of plasma adrenaline and hyperglycaemia after AMI may both activate platelets, and this could contribute to poor outcome in such patients.⁷⁵ In this study, the mean blood glucose level and HbA1C levels were significantly higher in the case group. However, in our two study populations of non-diabetic males there was no consistent relationships between blood glucose level or HbA1C levels and platelet aggregability.

7.5.4.7 Limitations. A number of important potential limitations need to be discussed, particularly given some unexplained and surprising associations. With multiple correlations, there are specific limitations and these associations may simply result from these well recognised problems. Nevertheless, other biases may similarly lead to unpredicted relationships.

1. Firstly, aggregation studies may be confounded by medications (especially aspirin), used by the study subjects. However, it is more probable that the patient group rather than the controls may have accidentally or surreptitiously used aspirin. Moreover, many common cardiovascular drugs in use appear to be able to reduce platelet reactivity.^{512,519,520} These possibilities would discriminate against an

increased platelet reactivity in the case group being detected. Nevertheless, despite the potential to bias against finding increased platelet reactivity in the CHD group, this was indeed observed.

2. The case group had on the average larger platelets (a significantly greater group MPV), and this may have resulted in an alteration in the aggregability of platelets. However, since the larger platelets in the case group may be partially lost during centrifugation, and since larger platelets may be more active, such an outcome would also reduce the likelihood of demonstrating a heightened platelet reactivity in the case group.

3. The unusual positive association between the apoprotein A1 and HDL levels and aggregation requires explanation. An obvious explanation cannot be provided from the data available in this observational cross-sectional study. The most likely cause is a statistical methodological bias and/or confounding factor. For example, the platelet population in the cases appears to be morphologically (and functionally) different from that in the control group. The removal of platelets resulting from aggregation (or because of their larger size) during preparation of PRP could result in a positive association between a "negative" risk factor, that is a factor with no relationship to platelet reactivity, and the remaining platelets.

4. In the current literature, the aggregation response to ADP has a stronger relationship with CHD than has been observed for collagen,^{901,902} thrombin⁹⁰² or adrenalin.⁹⁰² Our aggregation studies were not significantly different between the groups, except for adrenaline, and this observation differs from others.^{331,901,902}

5. The Hc and fibrinogen levels in the control group were associated with a reduction in aggregation, contrary to expectation. This again may be due to the preparation methods, since increasing amounts of fibrinogen and red cells in normals could cause more aggregation during collection and preparation of PRP. This in turn would result in reduced aggregation during *in vitro* testing. This suggestion is supported by the observed association between BTG levels measured in the same blood sample and platelet aggregability. The BTG level is a sensitive measure of the release reaction which may have occurred during the collection process.

7.5.5 Summary

1. Young males with premature CHD have different *in vitro* aggregation responses from normal controls, particularly in response to adrenaline.

2. These aggregation responses are influenced by different factors in the two groups. Aggregation in the control group was positively influenced by the platelet number in the plasma and negatively by the red cell volume in the blood. In the case group, aggregation was directly influenced by thromboxane metabolism, by the WCC and by apoprotein A1 levels.

3. The results provide further support for recent findings that platelet aggregation is predictive of cardiac events and CHD mortality. This concept is further strengthened by our observation that there is a significant relationship bet-

ween the severity of CAD and adrenaline-induced platelet aggregation in young males with premature CHD.

Table 7.20. Aggregation responses to adrenaline in the two study groups.

	Aggregation (%)	No Aggregation (%)	
Controls	33 (69)	15 (31)	Fishers' Test
Cases	46 (92)	4 (8)	p=0.0046 *

The numbers are the number of subjects in each group, bracketed numbers are the %.
Three aspirinated subjects in the case group have not been included.

Table 7.21. Threshold concentrations of agonists to achieve second phase aggregation.

	Concentration					
	12uM	25uM	50uM	100uM	1mM	No Aggreg
ADR Controls	6	9	10	1	7	15
Cases	9	11	7	8	11	4
						Chi-S=13.996 p=0.0156*
ADP Controls	1	14	21	4	2	
Cases	4	19	19	7	2	
						Chi-S=2.7899 p=0.7323
	9.5	19	28.5	38	47.5 (ug/ul)	
COL Controls	8	23	8	8	1	
Cases	18	22	7	4	1	
						Chi-S=5.1166 p=0.2756

Table 7.22. *In vitro* platelet aggregation responses in the two study groups (mean+/-SE).

	Controls	Cases	95% CI of Mean Diff		
LT50ADR (sec)	230 (n=33)	+/-170	130 (n=46)	+/- 70	156 to 44 p=0.001
LT50ADP (sec)	27	+/- 14	31	+/- 15	-2 to 1 p=0.162
LT50COL (sec)	138	+/- 69	121	+/- 73	-45 to 11 p=0.233
RADR (cm/min)	8.6 (n=33)	+/- 4	13.9+/- 8.8 (n=46)		2.0 to 8.6 p=0.002
RADP (cm/min)	27.8	+/- 15.4	32.1+/-26.2		-4.2 to 15.5 p=0.323
RCOL (cm/min)	23.4	+/- 12.5	31.2+/-24.0		7.3 to 15.5 p=0.048
LAGCOL (sec)	82	+/- 55	66	+/- 44	-36 to 4 p=0.109

Table 7.23. Correlations with platelet aggregation measures in the two study groups.

	TXB2	BTG	PCR	MPV	FIBRIN
A. Control Group:					
LAGCOL	-0.571~	0.533~	0.160	-0.058	-0.061
LT50ADR	-0.398*	0.521~	0.270#	-0.082	0.141
LT50ADP	-0.176	-0.051	0.025	-0.140	0.334*
LT50COL	-0.431~	0.614~	0.219#	-0.143	0.087
RADR	0.168	-0.360#	-0.221#	0.050	-0.356#
RADP	0.345*	-0.326*	-0.197	-0.021	-0.331*
RCOL	0.330*	-0.455~	-0.295#	-0.011	-0.157
B. Case Group:					
LAGCOL	-0.471~	0.090	0.343*	0.179	-0.190
LT50ADR	-0.125	0.355*	0.012	0.062	-0.076
LT50ADP	-0.109	-0.038	0.047	0.023	-0.022
LT50COL	-0.527~	0.039	0.255#	0.246#	-0.109
RADR	0.331*	-0.448~	0.157	-0.200	0.145
RADP	0.130	-0.117	0.027	-0.072	0.012
RCOL	0.090	0.046	-0.140	-0.052	-0.276#

Abbreviations are detailed in Chapter 2 and Tables 7.1 and 7.2.

Table 7.24. Correlations between platelet aggregation measures and CHD risk factors.

	CHOL	TRIG	HDL	APOA	APOB
A. Control Group:					
LAGCOL	-0.053	0.164	-0.254#	-0.173	0.197
LT50ADR	-0.056	0.044	0.099	-0.095	0.197
LT50ADP	0.003	-0.040	0.096	0.140	0.030
LT50COL	-0.063	0.155	-0.263#	-0.243	0.199
RADR	0.096	-0.213	0.081	0.269	-0.091
RADP	0.030	-0.082	0.244#	0.362*	-0.260
RCOL	0.208	-0.147	0.278#	0.252	-0.019
B. Case Group:					
LAGCOL	-0.147	-0.025	-0.041	0.019	-0.185
LT50ADR	-0.272#	0.140	-0.220	-0.447*	-0.162
LT50ADP	-0.034	-0.057	0.155	0.035	0.029
LT50COL	-0.164	0.185	0.047	-0.027	-0.203
RADR	0.421*	-0.139	0.266#	0.501~	0.352#
RADP	0.266#	-0.161	0.280#	0.572~	0.153
RCOL	0.125	-0.071	0.261#	0.370*	0.077

Table 7.25. Linear correlations between platelet aggregation measures and risk factors for CHD.

	DBP	HbA1C	Hc	WCC	PRPPC
A. Control Group:					
LAGCOL	-0.235#	0.140	-0.235#	0.190	-0.269#
LT50ADR	-0.288#	-0.030	-0.286#	-0.194	0.178
LT50ADP	0.081	0.291#	0.165	-0.098	-0.259#
LT50COL	-0.173	0.124	-0.419~	0.111	0.193
RADR	0.229	-0.192	0.311#	0.199	0.259#
RADP	0.244#	-0.093	0.191	0.081	0.244#
RCOL	0.195	-0.144	0.310*	0.053	0.065
B. Case Group:					
LAGCOL	-0.007	-0.283#	-0.142	-0.350#	-0.327*
LT50ADR	0.111	0.054	0.049	-0.069	-0.086
LT50ADP	0.143	0.273#	-0.091	-0.181	-0.375*
LT50COL	-0.058	-0.187	-0.118	-0.298#	-0.374*
RADR	-0.175	-0.061	0.013	0.152	0.389*
RADP	0.064	-0.133	0.042	0.129	0.254#
RCOL	0.172	0.058	0.021	0.099	0.273#

p<0.05, * p<0.02, ~ p<0.005

Abbreviations are detailed in Chapter 2 and tables 7.1 and 7.2.

Table 7.26. Regression analysis with measures of platelet aggregation as the dependent variable, and serum TXB2, PRPPC, WCC, Hc, Fibrinogen, DBP, Apoprotein A1 and Cholesterol values as the independent variables.

Dependent Variable	Independent Variable	B	SE B	Beta	R sq	P value
A. Control Group:						
LAG	Hc	0.006	0.001	0.852	0.353	0.0001
	FIBRIN	0.003	0.001	0.521	0.556	0.0064
LT50COL	Hc	0.005	0.001	0.722	0.521	0.0001
RCOL	Hc	-0.007	0.001	-0.842	0.501	0.0000
LT50ADR	Hc	0.005	0.001	0.744	0.553	0.0010
RADR	PRPPC	0.002	0.001	0.702	0.429	0.0025
LT50ADP	PRPPC	-0.001	0.002	-0.561	0.439	0.0021
	FIBRIN	0.001	0.001	0.400	0.589	0.0196
RADP	Hc	-0.003	0.001	-0.429	0.268	0.0252
	PRPPC	0.001	0.001	0.382	0.406	0.0438
B. Case Group:						
LAG	WCC	-1.306	0.580	-0.441	0.194	0.0352
LT50COL	TXB2	-0.379	0.091	-0.688	0.311	0.0005
	APOA	-0.002	0.001	-0.457	0.502	0.0117
RCOL	APOA	0.005	0.001	0.640	0.321	0.0007
	PRPPC	0.003	0.001	0.437	0.506	0.0128
RADR	APOA	0.005	0.001	0.625	0.390	0.0032
RADP	APOA	0.006	0.002	0.626	0.391	0.0014

Only variables retaining a significant association are reported.
B=regression coefficient, Rsq=coefficient of determination, SE=standard error of B, Beta=beta coefficient. Other abbreviations are detailed in Chapter 2 and Tables 7.1, 7.2, 7.2 and 7.6.

Table 7.27. Correlation between a measure of adrenaline-induced platelet aggregation (LT50ADR) and coronary angiographic scores.

Variable	r-value
Normal	0.235
CSS	-0.206 *
mCSS	-0.284 #
>50%	-0.170
>70%	-0.198
Jenkins	-0.318 #
CAS	-0.134
mCAS	-0.187
CS	-0.237
LVS	-0.297 #

p<0.05, * p<0.02 .Abbreviations are detailed in Tables 7.6.

7.6 SUMMARY

In summary, comparing the two study groups, there is *a significant difference in measures of inducible platelet function*, that is the PCR, serum TXB2 formation and adrenaline-induced platelet aggregation, but not in *in vivo* measures of platelet function, namely plasma BTG and plasma TXB2 levels (Table 7.28). These differences provide the most reliable evidence for a difference in this study.

Support for a difference in platelet function is also contributed to by the difference in the factors correlating with platelet function tests, although because of the multiple comparisons, these observations are less reliable. The second avenue of confirmatory support comes from the association of some measures of platelet function with angiographic measures of CAD. These will be further discussed in Chapter 11.

There appears to be sufficient evidence to indicate that differences in inducible platelet function may be a risk factor as defined by the presence of a measureable relationship with the disease and a difference compared to a group without the condition.

It is possible that a common group of post-AMI patients exist who are at increased risk of death or recurrent non-fatal MI,⁸⁰⁶ and who can be identified by an increased positive spontaneous platelet aggregation,⁵⁹ increased *in vitro* platelet aggregation,^{901,902} an increased platelet release reaction,⁷⁶⁸ more reactive platelets,⁸⁰⁶ or other methods of measuring increased platelet reactivity.⁴⁶

Table 7.28. Comparison of the group means of the platelet function tests for the case and control groups (mean+/-SE)

	Controls	Cases	95% CI Mean Diff
PC (10 /ul)	254 +/-7	245 +/-8	-30.28 to 12.28 p=0.40
PRPPC	325 +/-12	294 +/-11	-63.2 to 1.2 p=0.05
MPV (fl)	8.88+/-0.14	9.4 +/-0.1	0.18 to 0.86 p=0.003 *
PCR	0.87+/-0.01	0.81+/-0.02	-0.11 to -0.01 p=0.01 *
Serum TXB2 (ng/ml)	61 +/-9	111 +/-12	0.120 to 0.40 p=0.0004 *
Plasma TXB2 (ng/ml)	ND	ND	
BTG (ng/ml)	40.3 +/-2.5	47.0 +/-5.1	NS

PC= platelet count, MPV=mean platelet volume, TXB2=thromboxane B2, BTG=beta-thromboglobulin, PCR=platelet count ratio, ND=not detected, NS=not significant.

CHAPTER 8

THE DIFFERENT PREDICTIVE VALUE OF RISK FACTORS FOR ANGIOGRAPHICALLY DEFINED CORONARY ARTERY DISEASE IN MALES WITH PREMATURE CORONARY HEART DISEASE

8.1 VALIDATION OF THE CORONARY ANGIOGRAPHIC SCORING SYSTEM

8.1.1 Introduction

Coronary angiography is a technique in which a direct intra-coronary injection of contrast medium allows the radiographic visualisation of the coronary arteries. This technique was first reported by Mason Sones and his colleagues in 1959⁹²² and still remains the definitive procedure for delineating the coronary arteries and demonstrating the distribution and severity of coronary atherosclerosis in living humans.⁹²³ The primary goals of coronary angiography are the identification, localisation and assessment of disorders of the coronary arteries, primarily coronary atherosclerosis. A secondary goal is to assist in determining whether or not a patient is a suitable candidate for any of the various forms of revascularisation, such as coronary artery bypass surgery.⁹²⁴ Coronary angiography has been extensively utilised in the epidemiological study of CAD,⁶²³ allowing not only the evaluation of CAD based on the clinical manifestations, but also a measure of coronary atherosclerosis as measured by this technique. Coronary artery atherosclerosis is a diffuse disease which also consists of localised intra-luminal obstructive lesions representing the most severe manifestation. At present, coronary angiography is used predominantly to identify and quantify the more severe segmental disease. Three main approaches for identifying and quantifying severity of CAD are generally used.^{612,613,617} Firstly, functional measures of severity, such as the pressure gradient-flow ratios and coronary flow reserve are becoming more useful. Second, and most commonly, anatomic and geometric measurements on angiograms are the mainstay of assessment. Finally, indirect indicators, such as thallium perfusion scanning, provide valuable clinical information particularly related to functional myocardial perfusion.

These measures concentrate on the effect of the stenosis with respect to the encroachment into the lumen. However, the study of the extent, as well as severity of disease is necessary in order to develop an understanding of the pathophysiology and the natural history of CHD. Identification of the clinical, functional, biochemical, molecular and anatomical factors that influence plaque stabilisation have important clinical consequences.⁶²² Such factors may determine whether an area of atherosclerosis remains an innocuous intimal process or becomes a life-threatening lesion. The development of generalised atherosclerosis may merely be one contributing factor for the development of clinical CHD, with other factors required to change the lesion acutely into a significant obstruction. Indeed, the formation of acute severe stenoses may have different risk factors and mechanisms than that for the development of diffuse intramural coronary atherosclerosis.^{4,623}

8.1.2 The Interpretation and Limitations of Coronary Angiograms

The clinical utilisation of coronary angiograms involves cardiologists or radiologists inspecting coronary artery images projected at a 2-3 times magnification. The disease location is defined in terms of arterial anatomy, and the disease severity is specified as the "percentage stenosis".⁹²⁵ The percent stenosis is the percentage of segmental reduction in lumen diameter relative to an appropriate nearby "normal" diameter. The assessment of severity and distribution of lesions are coupled with

judgment as to the adequacy of the distal vascular bed for bypass surgery.⁹²⁵

There are serious limitations in this subjective assessment of disease severity^{611-613,619} and a number of these are outlined below:

1. *Considerable inter- and intra-observer variability exists even for experienced clinicians,*⁹²⁶ with up to a 35% variance among experienced angiographers evaluating proximal lesions with greater than 50% stenosis.⁹²⁷ There are also significant errors in specifying disease location.⁹²⁵ Some consistency of evaluation has been found with a four person panel of internationally recognised experts rendering a "consensus opinion",⁹²⁵ a situation that is not practical for normal clinical or epidemiological work. Even with such ideal reporters, clinically very important disagreement occurred in 2/14 cases regarding even the left main stem artery.⁹²⁵ Quantitative evaluation of the accuracy of an individual's and a panel's visual interpretations of coronary arteriograms have highlighted substantial inaccuracies.⁶¹⁸ It has been proposed that arteriographic interpretations accurate enough for decisions of major importance can only be made using quantitative arteriography, or at least the mean value of data from a large panel of angiographers.⁶¹⁸

2. *Coronary angiography also seriously underestimates the extent and severity of CAD.* The majority of careful studies correlating angiographic findings and the pathology show important discrepancies.⁹²⁸ Correlations with the anatomic pathology and physiological estimates have revealed that haemodynamically important lesions may not be recognised angiographically and less important lesions can be over-estimated.⁹²⁹ Even "normal" areas used as a baseline may be diseased.^{928,930,931} Furthermore, many persons have an adequate, if not normal, luminal cross-sectional area in the presence of advanced atherosclerosis.⁶²² A possible mechanism for this is the compensatory enlargement in stenotic coronary artery segments in the early stage of CAD.⁹³² In addition, biological variations of the coronary arteries are a major source of error.⁹³³ A significant degree of morbidity has been related to the inexactness of the appraisal of severity.⁹²⁸

3. *No angiographic quantitative method of assessment of the coronary arteries adequately measures intramural atherosclerosis.*^{619,934} Alternative methods are now being sought.^{611,612,934-936} Even though measurements from angiograms can be performed more objectively with advanced imaging technology assisted by computers,⁶¹¹ provision of suitable quantitative angiographic methods for epidemiological and clinical measurement are still awaited.⁶¹⁹

4. *The difficulties described above have also been highlighted by interventional studies using angiographic followup.* Even though angiography has been used for interventional risk factor studies^{937,938} and the study of therapeutic mechanical interventions,⁹³⁸ numerous problems exist with the utilisation of angiography in the evaluation of CHD risk factors,^{623,939} and even more so when applied to risk factor interventional studies.^{546,939,940} Any study using sequential angiography should take into consideration the potential for misdiagnosis of a change by single reading and that relevant quality control be formulated to determine reproducibility.⁹⁴⁰ In addition, clinical events related to thrombosis may lead to a rapid and significant change in severity unrelated to previous atherosclerotic disease severity.⁹⁴¹ Indeed there may

be no relation between progression of CAD and either the severity of stenosis on the initial coronary angiography or the elapsed time period.⁹⁴²

8.1.3 Quantitative Analysis of Coronary Angiograms

The clinical and investigational requirement for appropriate quantitative coronary angiograms has long been recognised,^{943,944} and the search for clinically relevant and suitable methods continues.^{616,617,619,945,946} Quantitative analysis of coronary angiograms ideally should provide absolute measurement of reference ("normal") and stenotic diameters, extent and asymmetry of the obstruction, relative percent diameter and area stenosis, the area of the atherosclerotic plaque, the roughness of the arterial segment, mean diameters of normal coronary segments, with all assessed from multiple projections.^{612,614} In addition, the relative and absolute cross-sectional narrowing, the functional pressure-flow effect of the stenosis and the coronary and stenosis flow reserve ideally should be measured as part of the clinical evaluation.^{612,614,617} The study of coronary atherosclerosis with these measures requires further evaluation with pathological and epidemiological correlates.⁹²⁹ This is exemplified by observations that angiographically "normal" segments of the coronary artery are not necessarily free of atherosclerosis,^{611,929,930,935} and, from a functional view point, that coronary stenotic lesions of proximal arteries reduce resting flow before distal arterial lesions.⁹⁴⁷

Currently a number of different techniques are available for the quantitative geometric and morphological densitometric computer-aided analysis of coronary obstruction and atherosclerosis.^{614,923,929,935,946,948} The equipment, procedures and computer software required currently are complex and are still being refined for more general clinical use.^{614,619,923,929,935,946,948} Pathological validation of automated videodensitometric and geometric computer algorithms using subtraction angiography has shown good correlation with histopathology, much better than group visual evaluation.⁹²⁹ Videodensitometry can provide an index of coronary luminal area and it would appear that results are independent of angiographic projection and luminal shape.⁹³⁵ Geometric measures can determine the absolute size of the coronary lumen. Absolute luminal sizes determined with geometric measures correlate better with the physiologic significance of a coronary artery stenosis than does percentage stenosis.⁴³ Computer based quantitative angiographic determinations of stenosis severity more accurately represent true luminal measurements,^{614,617} have closer correlation with indices of functional myocardial ischaemia, such as perfusion defects on thallium imaging,^{615,949} and provide various measures of coronary flow reserve.^{615,948}

These methods are currently incomplete, heterogeneous, and are undergoing continuous validation because of the known limitations.^{614,950-952} Although the methods are beginning to have practical applications,^{947,952} use for clinical and epidemiological studies tend to be limited and this has resulted in a search for more appropriate methods.^{931,934,953} Current readily available quantitative coronary angiographic techniques only address the severity of discrete segments of an artery and not diffuse CAD.^{612,619} Simple individual lesion measurements are able to be performed quickly and accurately on commercially available digital angiographic software packages. Evaluation of all angiographically visible atherosclerotic involvement of the arterial

tree by such methods would be extremely laborious and are as yet not practical,⁶¹¹ although potentially useful methods are being developed.⁶¹⁹ Moreover, significant variability of the accuracy of visual and quantitative techniques, albeit less so for the latter, exists in comparison to pathological measures.⁶²² A number of other lesions such as thrombus and myocardial bridges may mimic atheroma, as angiographic visualisation is an indirect technique.^{623,952} Such problems remain major unresolved difficulties for coronary angiographic use in epidemiological studies.⁹⁴⁵

8.1.4 Coronary Artery Disease Scoring Systems

The traditional method of risk stratification in CAD using coronary angiography has been primarily based on the number of diseased arteries and left ventricular function.^{620,621} Other methods of improving this prognostic information have been proposed,^{924,954} but not generally utilised as similar indices do not appear to substantially increase the prognostic information.⁶²¹ However, quantitative coronary angiography may contribute independent prognostic information.⁹⁵⁵ An extent score providing information about the minimal or moderate lesions may provide additional prognostic information over the conventional angiographic studies.⁶²¹ Even though the prognostic implications of an extent score for CAD are altered by the development of AMI or unstable angina, the extent of CAD always remains predictive of survival using Cox multivariate analysis.⁶²¹ A mean initial extent score has been reported to be higher in those with progression than without, and a greater extent score was found in those with multifocal progression.⁶²¹

8.1.5 Methods of Validation

To validate any angiographic technique a number of standard procedures are recommended which are basic to many laboratory methods:⁶¹⁴

1. Determine how large are the intra and inter observer variability from true values, using *ex vivo* and *in vivo* methods.
2. Determine the variability in repeated measures from the true value.
3. Determine the variability of measurement of the same arterial lesions repeated over time.

Such validation requires post-mortem material. Alternative methods, such as high-frequency epicardial ultrasound measurement, intra-coronary ultrasound measures, animal studies and/or *ex vivo* phantom studies for truly quantitative measures may be more exact.^{614,931,934,952,953}

The scoring systems used in the current study are not quantitatively derived and a visual estimation has been used. As discussed, the limitations of such techniques have been well defined.^{617,926,945} *Validation of the semiquantitative method in this study will involve assessing:*

1. Variability in the measurement of the same obstruction on two occasions separated by 6 months and made by the same observer blinded to the identity of the angiogram.
2. Comparison between different and validated angiographic scoring sys-

tems used in the literature.

3. Comparison of observations and results with other studies using the same or similar scoring methods.

4. Observation of predictable associations of the scores with known related factors, such as a relation with age and other established risk factors.

It is acknowledged that the utilisation of quantitative methods would be preferable, and that such methods should be applied in similar studies when they are available. The original report²⁰⁷ describing the scoring methods employed in this study demonstrated that the scoring system differentiated diffuse coronary atheromatous from distinct stenoses, "a prerequisite for the analysis of differences in aetiological mechanisms".²⁰⁷ This form of differentiation by quantitative angiography requires further development of computer assisted digital methods before being used as a research or clinical tool.

8.1.6 Description of the Scoring Methods

8.1.6.1 Coronary arterial segments and lesion assessment. Coronary angiograms were graded to give a measure of the severity and extent of atherosclerotic disease by a previously described semiquantitative method, developed and validated by Hamsten and colleagues.²⁰⁷ The coronary arteries were divided into predefined segments⁹⁵⁶ for measurement of the angiographically defined CAD. The CAS was used as a semiquantitative measure of the extent of involvement of the coronary arterial wall and the CSS was used as a measure of the cumulative severity of all distinct stenoses. Segments located distal to a total occlusion in the absence of sufficient post-stenotic contrast filling were not evaluated, nor were segments of hypoplastic arteries. The sum of all the CAS and CSS were also divided by the number of segments involved since the number of segments for each patient varied. This gave mean CAS (mCAS) and mean CSS (mCSS).

8.1.6.2 Grading diffuse atherosclerosis. The definition of angiographic atheromatous involvement was that described by Hamsten and colleagues.²⁰⁷ Atheromatous lesions in each segment were given a score for the extent of vessel wall involvement as defined angiographically and another for the degree of intrusion into the arterial lumen (Table 8.1). These 2 scores were then multiplied to produce an atheromatous segmental score as a measure of the extent of involvement within that segment. The CAS consisted of the total sum of each segment's atheromatous score for all the arterial tree.

8.1.6.3 Grading of lesion severity. The reduction in the vessel lumen diameter was estimated visually of all the stenotic lesions in each segment. The scores assigned are detailed in Table 8.2. The CSS is the sum of the segmental severity score for the whole arterial tree defined angiographically

8.1.6.4 Left ventricular function assessment. The left ventriculogram in the left anterior oblique view was divided into 5 segments.⁹⁵⁶ Normal contractility was scored as 0, hypokinetic wall motion as 1, akinetic wall motion as 2 and dyskinetic wall motion as 3. Each segment was scored and the total summed to give the left ventricular score (LVS), being a measure of LV wall motion abnormalities.

8.1.6.5 Arterial collateral assessment. The collateral score (CS) refers to the angiographic filling of any occluded artery. The occluded artery was divided into segments in the same way as that for the coronary artery scoring.^{207,956} If collaterals appeared but failed to opacify the occluded segment, the score was 1, for partial filling the score was 2 and for full opacification the score was 3. The segment scores were added to give the total collateral score. The intra-observer coefficient of variation for the angiographic scores varied between 2% and 7%, and all the angiograms were scored by the one observer.

8.1.7 Angiographic Procedural Methods

Cardiac catheterisation was performed by the percutaneous transfemoral technique using Cordis F7 sized catheters. The left ventriculogram was obtained at the beginning of the study. Coronary angiography was recorded on 35mm cine film at a film speed of 50 frames per second. The coronary arteries were examined in both the right (RAO) and left anterior oblique (LAO) views, at shallow and steep angles, in the lateral projection and in the LAO and RAO with craniocaudal angulation. In addition, different views were taken when necessary to visualise non-tangential and overlapping segments. No nitrates were administered during the procedure. The cine films were viewed a second time, using the same Tagarno projector, by the author who was blinded to the identification of the repeat angiogram film, 6 months after the initial scoring.

8.1.8 Results

8.1.8.1 Repeated measures. No significant variation existed between the values (precision) of all the repeated measures (Table 8.3). Furthermore, no significant difference existed between the SD of 2 different observations separated in time by the one observer. The mean differences of the repeated measures were also small (Table 8.4, Table 8.5, Table 8.6). The repeated analysis did show a wide range of variability depending on the score concerned, although all showed sufficient accuracy to be clinical useful (Table 8.6). All the repeated measures correlated well with the initial observations indicating satisfactory reproducibility (Table 8.3). That is, the intra-observer variation for the scores on 2 separate scoring times of the same films has correlation coefficients (r value) from 0.83 to 0.98 (Table 8.3). Variability in the percent mean difference (accuracy) of the angiographic scores ranged from 0.3% to 2.4% (Table 8.6). Values for the standard error of the differences in the angiographic scores varied from 0.6 to 1.6 (Table 8.6), values comparable with the reproducibility of quantitative measures.⁶¹⁴

8.1.8.2 Biological correlates. The scores had a predicable relationship with age in the population groups studied (Tables 5.3, 5.4, 5.5 and 7.2). This relationship with age was greater for the extent of CAD than for the severity, an observation consistent with pathological studies showing an increase in extent of atheroma with age. In addition, age retained a significant relationship with CAS in multivariate analysis even in the younger study group with a more narrow age spread, and more so in the population with a broader age range (Tables 5.3, 5.4, 5.5 and 7.2). This aspect was discussed in detail in Chapter 5.

8.1.8.3 Comparison with previous studies. The correlations found in the CHD patients with the measures of CAD severity and extent, in general are consistent with those reported in the literature (Table 8.7). Age is uniformly associated with the extent of disease and with the Jenkins score. In addition cholesterol has been found to be uniformly associated with extent scores and the Jenkins score, in agreement with our observations. Apoprotein B values correlated with extent and Jenkins scores, again similar to our results. Apoprotein A1 values and BMI were generally unrelated to angiographic measures of CAD. The use of tobacco has been assessed by different methods, and not surprisingly the findings have been diverse. However, a study⁵⁹¹ documenting packyears found a relationship in agreement with ours, namely that there is a univariate relationship with the Jenkins score. HDL cholesterol and triglyceride levels varied considerably between studies, ours being similar to some and different to others (Table 8.7). Measures of extent consistently related to age, cholesterol and apoprotein B levels. Severity measures were more varied, supporting the possibility that factors other than the traditional risk factors may make a predominant contribution to the formation of severe lesions.

8.1.9 Discussion

The utilisation of coronary angiography as an epidemiological tool in the study of coronary atherosclerosis has acquired significant attention since its inception, and particularly over the last decade.^{623,952} The quantitation of coronary artery stenoses has made significant advances^{614-618,946} in parallel and of necessity with advances in computer technology and digitalisation of analogue information.^{614,616,617} However, from making the initial decision for this study to discriminate between discrete coronary artery stenosis and the extent of atherosclerosis involvement of the arterial tree, to the selection of a rationale and established scoring system,²⁰⁷ then to the initial presentation of data⁹⁵⁷⁻⁹⁵⁹ and to the formal acceptance of fully reviewed articles,^{61,298} there has been little progress in finding a suitable quantitative angiographic correlate with pathological CAD extent⁵⁹³ until very recently.⁶¹⁹ As a result much effort has been put into finding alternative methods of assessing intramural atherosclerosis, particularly using intravascular ultrasound.^{931,934,953}

8.1.9.1 Justification of Scoring System Used. The semiquantitative visual scoring method was used in this study because of a variety of reasons: (1) Such methods are currently more applicable to clinical and widespread epidemiological use; (2) The availability of the computer software, the methodology and equipment required for quantitative methods; (3) To quantitatively examine the whole of the arterial tree is not a practical proposition at present. Furthermore, no commercial packages are available,⁹⁴⁵ a difficulty which will probably be overcome in due course;⁶¹⁹ (4) Severe lesions and extensive disease are not limited to proximal large segments, and distal segments have been assessed less frequently by quantitative methods; (5) Disease extent has an independent adverse prognostic influence and also a positive relation with disease progression, independent of disease severity. This results in a different natural history for localised stenotic lesions and more extensive disease, emphasising the importance of measuring disease extent;⁶²¹ (6) Visual and quantitative methods have given similar results in the Leiden intervention trial.⁹⁶⁰

A major flaw affecting angiographic methods is illustrated by the observation that,

even when using quantitative techniques, a mean decrease in diameter is greater for a normal reference diameter than for a stenoses diameter or for overall mean values.⁶²¹

Examining the accuracy and precision of quantitative methods will provide a baseline or standard by which to compare methods such as that used in this study. Variability in the mean difference (accuracy) of the percentage of the diameter of the stenosis for quantitative angiography ranges from 0.1% to 3% and the values for the standard deviation of the differences (precision) in the percentage of the diameter of the stenosis varies from 2.5% to 6.2%.⁶¹⁴ Intra-observer variation on 2 consecutive (same) frames has a correlation coefficient (r value) of 0.98 for the quantitative analysis for cross-sectional area measurement. For the percentage area stenosis the intra-observer variation is approximately 2.6% with an r value of 0.99.⁶¹⁴ The validation of most systems is still incomplete and individual results are described in different terms.⁶¹⁴ These observations illustrate that scores using summation methods can have results with reproducibility similar to quantitative methods.

Since neither a large panel of observers nor satisfactory methods of quantitation of diffuse disease are available, the choice of a semi-quantitative measure with one reader was made in this study. Despite the inherent limitations of the method chosen, the accuracy and validation of the measurements appear to be acceptable for an experienced angiographer. Nevertheless, in the final analysis, the method used in this study is not ideal. The importance of more accurately defining the atherosclerotic process in the coronary arteries remains an objective for all researchers in this area. However, the eventual method employed may not be angiography, an investigation more appropriately called a "luminogram",⁹³¹ but a method that evaluates the wall of the artery as well as the lumen.^{931,934}

8.2 ANGIOGRAPHIC EVIDENCE SUPPORTING THE EXISTENCE OF DIFFERENT STAGES IN THE PROGRESSION OF CORONARY ARTERY DISEASE

8.2.1 Angiographically Defined Coronary Artery Disease

8.2.1.1 Predictability of progression of CAD. It is well established that CAD begins at a young age in predisposed people and may progress rapidly in a significant proportion of these individuals.⁹ Lesion progression is only partly related to the initial degree of narrowing.⁹⁶¹ The unpredictability of the progression of coronary disease to severe lesions encroaching into the arterial lumen was demonstrated early in the history of the use of the coronary angiogram.⁵⁵³ Even though the timing of progression is difficult to predict for an individual, there appear to be common sites of progression which can be more easily anticipated,^{551,553} and all grades of intraluminal narrowing increase over time.⁵⁶² Progression prone areas of CAD in the coronary epicardial arteries have also been demonstrated in autopsy studies of the young.^{962,963} Despite being able to say which areas of the vessel have a common site of progression,⁵⁵¹ the use of previous angiograms to predict progression in an individual has been unhelpful.⁵⁵² Not only does knowledge of the initial angiographically

defined pathology not permit accurate prediction of progression, the frequency of progression does not appear to be related to the initial number of vessels with greater than 50% luminal obstructions or luminal occlusions.⁵⁵² Lower progression rates in single vessel disease compared with two and three vessel disease have been reported.⁹⁶¹

8.2.1.2 Coronary occlusion. Coronary angiographic follow-up studies have shown that coronary arterial occlusion may be related to the degree of stenosis found in a first angiographic study independent of time,⁵⁶⁴ an impression that appears intuitively logical. Indeed, the possibility of finding a new occlusion in a second angiographic study is, in part, related to the severity of disease seen in a prior angiogram.⁵⁵¹ Others have shown that a new coronary occlusion may occur independent of time.^{551,564} Moreover, a new occlusion can not always be predicted by the severity of previous existing lesions.⁵⁵¹ Non-occlusive progression also occurs in relation to the severity of the initial segment as well as time, but again, the specificity for prediction in individuals is low.⁵⁵¹

8.2.1.3 Lesion morphology. It would appear, however, that disease severity and linear progression over time are not the only factors involved in CAD progression. Different lesion morphology also appears to be indicative of factors influencing CAD progression. An increased tendency for progression of tubular stenoses and ulcerating plaques has been documented.⁵⁶² All grades of intraluminal narrowing increase over time, possibly with milder lesions tending to progress more rapidly.⁵⁶² Even in these studies, knowledge of the initial pathology in the coronary artery does not permit reliable prediction of where new lesions will occur, and from previous angiography one is unable to predict accurately which vessels will be involved in any future progression.⁵⁵²

In conclusion, differences in progression cannot be solely explained by differences in the degree of narrowing of initial segments even in those studies where progression was demonstrated to be, in part, related to the initial degree of narrowing and the course of time.⁹⁶¹

8.2.1.4 Extent and severity of CAD. The presence of diffuse disease in the coronary arteries may be of some help in stratification as to the risk of progression in those with single vessel disease, but it appears to be of little help in those with two or three vessel disease.⁹⁶¹ The extent of CAD, in terms of the number of lesions less than or equal to 75% narrowing, may be related to the development of a new occlusion.²¹ The severity of these lesions was not clearly indicated, however, and the occurrence of progression and occlusion was also independent of each other.²¹ Indeed, CAD progression occurred with or without occlusion and vice versa.²¹ Using multivariate analysis, predictors of subsequent new occlusion derived from the first catheter included greater than 80% narrowing supplying a non-infarcted segment, the extent of coronary disease, cigarette smoking and the male sex.²¹ The majority of new occlusions, however, occurred in normal vessels or vessels with moderate lesions (less than 75% luminal narrowing).²¹

Severe lesions supplying normally functioning myocardium have been found to have a high rate of occlusion within the time interval between angiograms. Therefore very

severe lesions with more than 80% luminal narrowing may identify individuals with an increased likelihood of coronary occlusion. This is commensurate with the risk of re-occlusion in individuals treated with streptokinase for AMI⁴³ and the association between severe stenoses and subsequent occlusion in patients with unstable angina.⁵³⁴ Moreover, spontaneous obstruction of severe coronary artery stenoses amenable to PTCA is not uncommon and is time dependent (5% within an average of 76 days), although the prediction of a subsequent occlusion was not possible for an individual.⁹⁶⁴

8.2.1.5 Normal or 'near normal' coronary arteries. Those with normal coronary arteries at the time of an initial coronary angiogram appear to be unlikely to develop CAD by the time of a repeat catheter.⁵⁵³ Even though coronary arteries that are angiographically normal in individuals without CHD have a low rate of progression,⁵⁵³ those with some intraluminal disease without a significant luminal obstruction do have a rate of progression almost equivalent to those individuals with CHD.⁵⁵⁴ Even though progression occurs in old existing atheromatous lesions, new areas of significant narrowing frequently occur in previously normal segments⁵⁵⁵ and progression to occlusion is less common in pre-existing moderately severe lesions, unless the stenosis is greater than 90%.⁵⁵⁵ In another study, the majority of new occlusions (116) were reported to occur in angiographically normal vessels (17 out of 116) and with lesions having less than 75% luminal narrowing (66 out of 116).²¹ These observations provide further evidence of the low predictive accuracy of angiographic criteria to predict which coronary artery lesions will subsequently occlude the artery.

8.2.1.6 Significant progression from areas of minimal CAD. There is little doubt that CAD is progressive, increases with age, has a rate of progression proportional to the extent and severity of the underlying atherosclerosis, and commonly occurs within five to seven years.⁵⁵⁷ Also, as indicated, the progressive nature of the disease is quite episodic⁵⁵⁶ and progression to occlusive CAD occurs quite commonly in areas that have previously been minimally diseased in patients initially without clinical disease later requiring coronary angioplasty or CABG.^{555,557,558} A particularly important point that requires emphasis is that the majority of these studies surveyed populations that initially did not require revascularisation.

8.2.2 CHD Risk Factors and Progression

Information about the predictability of CAD progression in individuals, or groups, according to the presence or absence of risk factors is highly variable. Clinically useful and specific identifying factors indicating the risk and timing of progression are not clearly distinguished. These include the presence of a family history, obesity, hypertension, diabetes, an abnormal ECG and even the initial coronary angiogram.⁵⁵² A great deal of variability in the relationship between risk factors for coronary disease and progression has been demonstrated both in older and newer angiographic studies. Abnormal glucose tolerance, lipid levels and myocardial lactate production may correlate with widespread progression without any association with the age, sex or smoking habits of the patients nor the interval between studies.⁹⁶⁵ In early studies, others have found no relationship between disease progression and individual risk factors.⁹⁶⁶ Age has not universally been found to be a

strong predictor of rate of progression, although some studies have shown that the mean age of those with measurable progression is slightly lower than those without.⁵⁵² The inconsistencies would appear to be present even prior to the current era of intensive risk factor modification that has occurred since the 1980's in many Western cultures.

Cholesterol and triglycerides are related to the progression of CAD⁹⁶⁷ although usually a combination of risk factors must be present.⁵⁵² Recent reports have provided more convincing data that at least some lipoprotein levels have consistent predictability for CAD progression. In the Cholesterol Lowering Atherosclerosis Study (CLAS), a global angiographic score was utilised to assess progression, which is associated with total cholesterol, LDL cholesterol, non-LDL cholesterol, triglycerides, apoprotein B, and diastolic pressure by univariate analysis, but only with non-HDL cholesterol in placebo treated patients using multivariate analysis.⁹⁶⁸ These associations, both for the univariately and multivariately variables, are not helpful for predictive purposes in the individual. There is a weak association with progression and the ratio of total cholesterol or LDL cholesterol to HDL cholesterol levels.^{968,969}

Thus, the relationship and predictability of CAD progression with CAD risk factors requires much greater clarification and improved specificity, given the degree of disparity between studies.^{21,561,562,961} Despite the lack of specificity for CAD risk factors predicting progression, modification of these factors has been shown to have a beneficial and measurable influence on CAD progression. Improvement in the blood lipoprotein profile has been the main target for these studies.^{268,272,274,970-975} Multifactor intervention was also found to be of benefit.^{960,976} Other potential areas may include intervention into haemostatic factors,⁵⁶ psychological factors,⁹⁷⁷ and a combination of lipid and haemostatic variables.⁹⁷⁸

8.2.3 Time Dependence of Progression

Progression correlates with the duration between coronary angiograms,^{560,561} time being predictive of new occlusions.⁵⁶⁰ This time factor, however, is only one influence on the process. There are also time independent factor(s).^{551,564} Of particular relevance, dependence on time does not appear to be present for those developing unstable angina in comparison to those who have had stable angina and develop progression of their CAD.⁵⁶⁰

8.2.4 Clinical Symptoms and Progression of CAD

8.2.4.1 Stable symptoms. Clinical symptoms are considered to be indicative of CAD progression.^{560,966} However, angina is not necessarily predictive of progression of disease affecting all segments of the coronary artery.⁹⁶⁵

8.2.4.2 Acute ischaemic syndromes. The occurrence of an AMI and an increase in symptoms of CHD is associated with the degree of CAD progression.⁵⁶¹ Those with an acute infarct occurring between angiographic studies are more likely to show progression. Nonetheless, in the same group, progression was also common in those with symptomatically stable CAD and for those with improving symptoms.⁹⁶¹ Others

have found that only the proximal location of the stenosis was a significant predictor of infarction.⁹⁷⁹ Nevertheless, AMI due to total coronary occlusions frequently developed where previously non-severe lesions were present, and predicting the location of a subsequent AMI from a prior angiogram is difficult.⁹⁷⁹

The percent narrowing, involvement of the left anterior descending artery, the presence of eccentric and complex lesions, the number of diseased vessels and/or the presence of unstable angina are predictive of CAD progression.⁹⁷⁹ Nevertheless, in another study, the majority of lesions which were noted to progress concurrent with the development of unstable angina were initially narrowed less than 50%.²⁰ Clearly, mild to moderate lesions are subject to acute thrombotic occlusion^{980,981} and a substantial number of mild to moderate lesions may be associated with thrombosis and AMI.⁹⁸¹

8.2.5 Pathology and Progression

At least three important facts arising from autopsy studies of CAD and CHD highlight the fact that severe obstructive lesions develop intermittently and probably acutely.

Firstly, individuals with CAD usually have extensive disease by the time they present clinically. No difference in the number of major epicardial arteries having severe CAD has been found in those with a first anterior or posterior infarct, except in the frequency of the involvement of the left main stem coronary artery.⁹⁸² This observation is from a cross-sectional necropsy study of patients with a fatal first AMI.⁹⁸² Severe and extensive narrowing of the three major coronary arteries was present irrespective of whether the infarct involved the anterior or posterior section of the ventricle.⁹⁸² Moreover, individual variation in disease severity within the coronary tree was great. In addition, prediction of the most severely narrowed coronary artery, using the location of the infarct in those suffering their first and fatal AMI, was difficult.⁹⁸² In fact, necropsy studies indicate that CAD is extensive by the time of the first MI, irrespective of its location.⁹⁸²

Secondly, those lesions resulting in the clinical manifestations of CAD are not simply comprised of an accumulation of the components of atherosclerosis. Acute changes occur within the atherosclerotic plaque. A post-mortem angiographic study comparing histological findings demonstrated that lesions with angiographic features indicating a complicated lesion represent a greater risk for AMI or sudden death than do uncomplicated lesions.¹¹² This study suggested that stenoses characterised angiographically by irregular borders or intraluminal lucences probably have a more adverse clinical outcome. Plaque rupture without thrombosis formation was also documented,¹¹² a not uncommon finding in individuals dying of non-cardiac causes with and without CAD.²³⁸

Thirdly, anatomical studies have shown that a substantial percentage of lesions associated with coronary artery thrombosis and AMI are moderate in severity,^{12,559} confirming the angiographic observations discussed earlier.

8.2.6 Limitation of Angiographic Follow-up Studies

8.2.6.1 Selection bias. Patients are usually selected for a second coronary angiogram because of new symptoms or clinical events. However, a group of patients who were in a stable or improving clinical condition and elected for surgery treatment, consequently had a repeat routine angiogram.⁹⁶¹ Progression in this group was common, with relatively small differences compared to those who had a repeat cardiac catheter for clinical reasons.⁹⁶¹

The effect of survival selection may well lead to an underestimation of the amount and degree of progression. Significant disease found in an initial study will increase the likelihood of this bias as well as reducing the likelihood of those with severe disease coming to further angiography increasing. These factors will increase the likelihood that those with less severe disease will be detected as having progression more frequently than does in fact occur. However, the basis of symptom selection would also bias toward overestimating the frequency and degree of progression. The significance of this is uncertain in view of reported findings that those without symptom deterioration, and even those with symptom improvement, may also have lesion progression.⁵⁵¹

Another selection bias may occur when those with more severe disease have CABG or a PTCA, while those who have mild disease or severe inoperable disease will continue with medical treatment.

8.2.6.2 Interpretation error. Technical factors have also been provided major limitations in angiographic studies, as previously discussed, particularly in relation to the inter- and intra- observer variability for visual imaging.^{923,926} The interpretation of the coronary angiogram underestimates the severity of disease in those with severe lesions and both under and overestimates mild to moderate lesions.⁹⁸³ Diffuse coronary atherosclerosis is often present when coronary angiography reveals only discrete stenosis indicating that coronary angiography underestimates the severity and extent of CAD.⁹⁸⁴

8.2.6.3 The angiogram as a "luminogram". The coronary angiogram is not an ideal method of studying CAD because of the fact that the method only gives information on the lumen of the coronary artery. The limitations associated with this fact are discussed in section 8.1.

8.2.7 Summary

Assessment of CAD using coronary angiography is insensitive in predicting the time and location of a subsequent new lesion or progression to occlusion. In particular, it does not allow accurate prediction of the occurrence or location of a MI in individuals with mild to moderate disease.⁵⁵⁸ The high incidence of progression in vessels that are normally minimally diseased has been well documented.^{20,555,557,558,560} Patients with mild to moderate disease may develop progression more commonly in these areas,^{557,558} although the progression to total occlusion also occurs frequently in very severely narrowed lesions.^{21,43,555}

Clearly, the *"progression of coronary atherosclerosis is a highly unpredictable process that follows a non-linear course and that information about its dynamics derived from sequential coronary arteriograms hardly improves prediction of future progression"*.⁵⁶¹ The need to determine the factors that do predict new lesion development more precisely, and hence the occurrence of life-threatening cardiac events, should be a high priority in evaluating the causative mechanisms of CAD and CHD. Given the contribution of thrombosis to the development of clinical events, and possibly progression, examination of the factors associated with severe stenotic lesions is necessary, as is the differentiation of factors associated with the development of atherosclerotic disease and those leading to the formation of localised severe stenosis. One potential factor for the latter may be a thrombotic tendency in individuals with underlying CAD. This has been recognised for most of this century, but has only attracted general recognition recently.

8.3 ANGIOGRAPHICALLY DEFINED CORONARY ARTERY DISEASE AND THE PREDICTIVE VALUE OF CORONARY HEART DISEASE RISK FACTORS

This section deals with the potential of CHD risk factors to be differentiated in the case and control groups and to also be associated with different aspects of CAD, namely, the extent of involvement of the arterial wall and the quantity of severe stenoses.

8.3.1 Introduction

Semi-quantitative angiographic scoring systems have been used with the intention of discriminating between atherosclerotic disease involving the arterial wall and lesions encroaching into the lumen of the artery.^{61,207,593} In this study a complex scoring system has been used to examine the relationship between angiographic measures of CAD, the traditional risk factors for CHD and measures of platelet function which may also constitute CHD risk factors. Such methods have been used extensively to investigate risk factors for CHD, particularly in the evaluation of the relationship of lipoproteins and CAD, but have not always produced consistent findings. For example, (as discussed in Chapter 5) the majority of angiographic studies have demonstrated a positive relationship between total blood cholesterol levels and CAD^{206,207,220,365,592-594,600,601} although not all.^{596,602} An independent relationship with angiographically defined CAD and total blood cholesterol values is not maintained using multivariate analysis.^{206,207,590,593} This latter observation was confirmed in the current study (Chapter 5). A univariate relationship between apoprotein B and LDL cholesterol levels with CAD and CHD has been well established,^{590,592,594,596,597} and an independent relationship is preserved using multivariate analysis.^{206,207,590,593} Again, this finding has been reaffirmed in the current study (Chapter 5). The relationship, however, was due to an association with the extent rather than the severity of atherosclerosis, particularly when the angiographic classifications are separated sufficiently well by appropriate scoring methods and evaluated by multivariate analysis.^{206,207}

As discussed in Chapter 5, the scoring system developed by Hamsten and col-

leagues²⁰⁷ has specific advantages over other systems in separating the extent of involvement of the arterial wall and the influence of discrete severe or obstructed lesions. For example, multiple minor lesions within a segment of the coronary artery is not uncommon in addition to more severe stenoses and previous studies have included mild lesions in the stenosis score.^{151,590,596} In addition, the severity scoring system of Hamsten et al²⁰⁷ gives a maximum of 16 to a segment, so that in the presence of an occluded segment, minor lesions make no impact on the stenosis score for that segment, and therefore the stenosis scoring system of Hamsten et al²⁰⁷ is more likely to be related to factors, including thrombosis, which are involved in the development of severe and/or occluded lesions.⁵⁹³

8.3.2 Methods

8.3.2.1 Study population. The study population consisted the case-control group described in Chapter 3.

8.3.2.2 Angiographic, statistical and laboratory methods. All the methods are described in Chapters 2, 5, 7 and 8.1. Also, other factors that impacted on the observations and have been included in the analysis are described in Appendix 1, 2 and 3. These latter factors are the percent plasma content of fatty acids (Appendix 1), scapular skinfold thickness (Appendix 2), and a psychologically based hostility score (the MMPI hostility score, Appendix 3).

8.3.3 Results

8.3.3.1 Number of normal segments. For the number of normal segments, two platelet function measures (viz. TXB2 level in clotted whole blood and the PRP platelet count) correlated directly and the MPV correlated inversely. The lipid variables of cholesterol, apoprotein B and percent plasma myristic acid content correlated inversely with the number of normal segments. Of the other risk factors, age, the packyear of cigarette smoking and the MMPI hostility score also correlated inversely (Table 8.8). When the number of normal segments was entered into the regression equation as the dependent variable, and the MPV, TXB2, age, packyears of cigarette smoking, hostility score, apoprotein B and plasma myristic acid were the independent variables, only apoprotein B and myristic acid values remained significantly predictive (Table 8.10). The PRP platelet count and cholesterol levels were not related independent of the MPV and apoprotein B respectively, and had the potential for causing problems due to multicollinearity. Therefore they were not included in the regression model.

8.3.3.2 Coronary artery extent scores. No platelet function variable correlated with the angiographic score of extent of arterial involvement. Cholesterol and apoprotein B levels, and plasma myristic content correlated directly, and aracidonic acid content inversely with the CAS (Table 8.8). Similar associations were found for the mCAS except for the aracidonic acid. Also, HbA1C levels, scapular skinfold thickness, diastolic BP and systolic BP were directly related to the extent scores (Table 8.8).

CAS and mCAS were separately entered into the regression equation as dependent

variables and the scapular skinfold thickness, myristic and aracidonic acid content and the apoprotein B level were the independent variables. Only the myristic acid content and scapular skinfold thickness remained independently predictive (Table 8.10). The cholesterol level, HbA1c level and BPs were not independent of other variables and, again, had the potential for causing problems due to multicollinearity. Therefore they were not included in the regression model.

8.3.3.3 Coronary artery severity scores. Those platelet function variables, namely TXB2 and PRP platelet count, were inversely related to the CSS, and LT50ADR was inversely related to the mCSS. The MPV was directly related to both the CSS and mCSS (Table 8.8). The only lipid variable associated with either score was the plasma myristic acid content (Table 8.8). The HbA1C level, age, packyears of cigarette smoking and the MMPI hostility score had direct associations with the severity scores (Table 8.8).

With multivariate regression analysis, only the LT50ADR value remained predictive of the severity scores. The independent variables entered were TXB2, MPV, myristic acid content, age, packyears, hostility score and the LT50ADR (Table 8.10).

8.3.3.4 The Jenkins angiographic score. The platelet variables which correlated with the Jenkins score were the LT50ADR and MPV. Plasma myristic acid content and apoprotein B level were directly associated as well as the HbA1C level, age and packyears of cigarette smoking (Table 8.9).

With multiple regression analysis, the MPV and LT50ADR value remained independently predictive of the Jenkins score as the dependent variable. The independent variables were the LT50ADR, MPV, apoprotein B level, myristic acid content, age and packyears of cigarette smoking (Table 8.10).

8.3.3.5 Lesion stenosis scores. The number of individual lesions with a percent diameter luminal narrowing that approximated a range of stenoses, for example between 75% and 90% diameter narrowing, were also evaluated. The associations were similar to those of the cumulative scores, albeit with more overlap (Table 8.9). Of particular note was the association of platelet function measures and specific fatty acids with the more severe stenoses, and the lipid variables with the less severe lesions (Table 8.9).

8.3.3.6 Other angiographic scores. Only measures of platelet function, namely the LT50ADR and PRPPC, correlated directly with the left ventricular score (Table 8.9). As expected, there was a strong relationship between the number of AMI and the LVS (Table 8.9). TXB2 values and the MMPI hostility score correlated with the collateral score. The NYHA functional class for angina also correlated with the collateral score (Table 8.9).

When the LVS was entered into the regression model as the dependent variable, and the number of MIs, LT50ADR and the CSS as independent variables, the number of MIs and the LT50ADR value remained predictive of the LVS (Table 8.10).

These results provide an argument for different factors affecting different stages in the development of CHD. Factors related to platelet function are more predictive of the severity of CAD. Measures of lipid and fatty acid metabolism, and body fat distribution appear to be associated more with the extent of involvement of the arterial wall with atherosclerosis. These results depend strongly on the validity of the angiographic results and additional discussion of the potential pitfalls of such measures are addressed below. Moreover, support for different stages/processes in the development of CHD arises from the results presented. This observation is discussed in the context of other studies also supporting such a proposition.

8.3.4.1 Further evidence for the validity of the angiographic scoring methods.

Firstly, in this analysis *there are clinically predictable associations with the angiographic scores*. One argument for the validity of the semi-quantitative method for the angiographic scores of CAD severity and extent has been provided in the first section of this Chapter. Further evaluation of these scores and clinically relevant variables confers further strong supporting evidence for the reliability and utility of the angiographic measures. The relationship between the functional class for angina and the severity and collateral scores highlights the credibility of the scoring system, in conjunction, acknowledging the limitations of angiographic scoring methods.⁶¹¹⁻⁶¹³ The lack of association of the angina functional class with the extent scores also supports the reliability of the method. Moreover, the LVS being associated with the number of previous MIs was expected. The inverse association of age, cholesterol, and apoprotein B levels with the number of normal segments also strengthens the reliability of the observations. The fact that cholesterol measurements performed before the diagnosis had a stronger relationship with the extent of coronary atherosclerosis than the cholesterol measurement undertaken during the study further adds to the consistency of the associations. The positive correlation between age and the extent of atherosclerosis is well documented in the literature.^{207,593} The relationship with disease severity is less consistent, with some studies showing an association,^{61,591,600-602} while others have not shown any correlation.^{206,207,593}

Secondly, *there was a reliable degree of internal consistency for the various associations and the other scoring methods for assessing CAD*. Methods of assessing coronary angiograms vary greatly, and differences in results between studies may clearly relate to such methodological differences.⁹⁸⁵ To attempt to internally assess the scope of this problem, more than one scoring method was used in this study. There was internal consistency between the clinical and laboratory variables and the Jenkins score and the CSS. This was maintained for the breakdown into the number of lesion of varying severity.

Thirdly, *a potential bias can arise from the selection of subjects, particularly if there is little variation in the measured variable, leading to an increased likelihood of false negative results*. In this study, subjects with CHD were compared to a normal control group without CHD determined on clinical grounds and by exercise testing. The presence of coronary artery atherosclerosis was not excluded angiographically. This could potentially lead to false negative results due to dilution bias if some of the

controls had CAD which was not clinically apparent. Such dilution bias may result in no measurable difference being documented between the groups for some of the variables which are true risk factors for coronary atherosclerosis. These factors would then not be included in the assessment of the angiographic measures using multivariate analysis. Nevertheless, the case group evaluated did have a demonstrable relationship with the more commonly accepted risk factors, and in particular the apoprotein B level. Conceding the potential for dilution bias, the validity of a negative finding is enhanced by the consistency of those positive associations observed between the angiographic scores and the separate variables.

Another important area of bias relates to the sample size of the study populations. A type II statistical error may have led to any negative findings, missing a weak relationship because of an inadequate sample size. However, predictable differences and associations of the classic risk factors were able to be detected. That is, clinically meaningful and established differences and associations have been determined from the sample number used in this study. Weaker and less clinically relevant relationships may have been missed.

Another bias leading to false negative results is the potential for regression dilution bias resulting from over-adjustment for variables in multivariate analysis. This could result from lack of precision of measured variables compared to less significant measures. Also, the problem of multicollinearity (that is, variables measuring the same relationship but being identified as separate and independent factors) is well recognised in the type of analysis used.

Finally, as discussed earlier, there are serious limitations to the subjective assessment of angiographically defined CAD⁶¹¹⁻⁶¹³ and there have been significant advances in the quantitative assessment of CAD in recent years.⁶¹⁴⁻⁶¹⁸ There has been little progress, however, in finding a suitable quantitative angiographic correlate with the extent of pathologically defined CAD to be used in epidemiological studies,⁵⁹³ although valid clinically applicable methods are currently being developed.⁶¹⁹ Even though the risk of CAD has been primarily based on the number of diseased arteries and left ventricular function,^{620,621} *a score expressing the extent of CAD and providing information about minimal or moderate lesions may contribute additional prognostic information beyond the conventional angiographic studies.*⁶²¹ The validity of the scoring system used in this study appears acceptable,²⁰⁷ although the limitations of the method need to be recognised.^{593,611-613}

Appropriate end-points for disease assessment are necessarily required. Anatomic CAD may not be the right end point for the expression of some CHD risk factors.⁹⁸⁵ There is a high probability that risk factors influence different stages in the development of the disease.²⁰² This is part of the hypothesis being explored in this Thesis, and the attempted division into intraluminal and intramural disease may help to evaluate the existence of different risk factors for different stages in the development of disease.

8.3.4.2 Evidence for different stages/processes in the development of CHD. The results of the International Antiatherosclerotic Therapy (INTACT) study present a possible insight into the mechanisms of disease progression as observed in that

study.⁵⁴⁶ The INTACT study confirmed previous experimental studies demonstrating a significant reduction in newly formed coronary artery lesions (stenoses and occlusions) in patients on nifedipine compared to placebo, especially in the presence of early CAD.⁵⁴⁶ In addition, there is now good evidence for a number of modifiers, such as pretreatment with betablockers, and possible triggers, such as vigorous exertion, for the development of AMI.⁹⁸⁶ A proposed explanation will be briefly stated and elaborated on subsequently.

Angiographic studies have demonstrated that progression of CAD involves new lesion formation that is not entirely related to the underlying atherosclerosis, implicating additional mechanisms, possibly thrombosis. Pathological evidence supports the involvement of thrombus in atherosclerotic disease progression.^{34,539} Secondly, clinical syndromes (unstable angina and AMI) are associated with new lesion development, in association with a fissured plaque, and increased thromboxane production. Moreover, increased TXB₂ production occurs in whole blood clotting in the presence of provoked ischaemia (Chapter 4).²⁹⁸ Thirdly, nifedipine appears to reduce the development of new lesions⁵⁴⁶ and pretreatment with betablockers reduces the proportion of morning infarction.^{223,903,986} The results of this Thesis show that the combination of nifedipine and metoprolol reduces the amount of thromboxane produced during whole blood clotting *ex vivo* (Chapter 4). Also, the prophylactic use of aspirin in middle aged men reduces the incidence of non-fatal MI, a thrombotic process, but not the development of angina, which is possibly due to a non-thrombotic process.⁶⁹⁵ Finally, this angiographic study has demonstrated an independent relationship between platelet function measures and discrete stenotic lesions but not extensive intramural disease. A reduction in thromboxane production might reduce the likelihood of the production of those new CAD lesions that involve thrombosis.

Such a scenario implies that disease progression, particularly the formation of discrete lesions, has a major non-atherosclerotic component, namely thrombus formation. The brief review of disease progression discussed in the introduction to this Chapter, as interpreted from angiographic studies, supports this concept. Despite being able to say which areas of the vessel have a common site of progression,⁵⁵¹ the use of previous angiograms to predict which vessels in an individual will be involved in progression has not been reliable.⁵⁵²⁻⁵⁵⁴ Undoubtedly progression occurs in old existing atherosclerotic lesions. However, new areas of significant narrowing frequently occur in previously normal segments⁵⁵⁵ and the progressive nature of the disease is quite episodic.⁵⁵⁶ Indeed, progression of occlusive CAD occurs commonly in areas that have previously been minimally diseased in patients without initially clinical disease requiring PTCA or coronary bypass grafting.⁵⁵⁵⁻⁵⁵⁸ Anatomical studies have shown that a substantial percentage of lesions associated with coronary artery thrombosis and AMI are moderate in severity.^{12,559} In summary, a high incidence of progression of CAD in vessels that are normally minimally diseased has been well documented,^{20,555,557,558,560} although the progression to total occlusion also occurs frequently in very severely narrowed lesions.^{21,43,555} The progression of coronary atherosclerosis is a highly unpredictable process that follows a non-linear course and information about its dynamics derived from sequential coronary arteriograms hardly improves prediction of future progression.⁵⁶¹ The importance of determining the pathological components which contribute to the development of acute severe lesions, whether related to the destabilisation of atherosclerotic plaque,

increased thrombogenicity, and/or other intrinsic or extrinsic factors, is evident.

8.3.5 Summary

1. The observations discussed above make the results of the current study of considerable interest. The results indicate that the formation of acute severe stenoses have different risk factors and mechanisms than those involved in the development of diffuse atherosclerosis, as has been previously proposed.^{4,623}

2. The observations support the concept of different factors influencing different stages in the development of the clinical condition. In young males with CHD, specific factors are associated with the atherosclerotic component and other factors with the stenotic component, and some with both.

3. In young males with CHD, platelet function plays an important part in determining the severity of the CAD. Fatty acids, lipoproteins and body fat distribution play a major part in determining intramural involvement of coronary arteries by atherosclerosis, as defined angiographically. In particular, no platelet function measure was related to the extent of disease.

Table 8.1. Grading of Diffuse Coronary Atherosclerosis

	<u>Score</u>
<u>Extent</u>	
Angiographically normal vessel wall	0
1-2 angiographic plaques	1
>2 plaques located in one or several groups with normal intervening portions	2
>2 plaques producing continuous irregularities	3
<u>Mean plaque size</u>	
Slight indentation (<10% reduction in diameter)	1
Intermediate size indentation (10%-25% reduction of the vessel diameter)	2
Large plaque (>25% reduction of vessel diameter)	3

Table 8.2. Grading of Severity Score

	<u>Score</u>
Normal or lesions reducing lumen diameter <25%	0
Lumen diameter reduced 25%-50%	1
Lumen diameter reduced 50%-75%	2
Lumen diameter reduced 75%-90%	4
Lumen diameter almost occluded 90%-99%, but antegrade flow still present	8
Arterial occlusion with no antegrade flow	16

Table 8.3. Relationship Between Repeated Measures (Intra-observer)

Variable	Mean	SE	r*	p value
CAS1	18.65	2.59	0.9478	<0.000
CAS2	16.75	2.08		
CSS1	27.85	4.30	0.9745	<0.000
CSS2	27.60	4.23		
LVS1	2.80	0.39	0.9501	<0.000
LVS2	2.95	0.47		
Normal1	6.65	0.60	0.8297	<0.000
Normal2	6.05	0.54		
CS1	5.85	1.13	0.9565	<0.000
CS2	5.20	0.99		
Jenkins1	11.80	1.70	0.9261	<0.000
Jenkins2	10.80	1.55		

* Correlation between the first and second measures
r = the Spearman correlation coefficient.

Table 8.4. Mean Difference of Repeated Measures

Variable	Mean Diff	SD	95% CI Diff Mean	p value
CAS	1.90	4.35	-8.615 to 4.815	0.5702
CSS	0.25	3.77	-12.454 to 11.955	0.9671
LVS	-0.15	0.75	-1.088 to 1.388	0.8076
Normal	0.60	1.46	-2.233 to 1.033	0.4617
CS	0.65	2.10	-3.691 to 2.391	0.6677
Jenkins	1.00	2.81	-5.659 to 3.659	0.6664

Mean diff = mean of the difference between repeated measures.

CI diff mean = confidence interval of the difference of the mean.

Table 8.5. Absolute Mean Difference of Repeated Measures

Variable	Mean Diff	SD
CAS	3.7	3.8
CSS	2.9	3.1
LVS	0.5	0.6
Normal	1.3	0.9
CS	1.2	1.9
Jenkins	2.3	2.0

Absolute mean difference = mean difference of numerical values without considering whether positive or negative.

Table 8.6. Percent Mean Difference of Repeated Angiographic Scores

Variable	% Mean Diff	SE
CAS	1.8	1.5
CSS	0.4	0.6
LVS	-0.3	1.2
Normal	2.3	1.6
Jenkins	2.4	1.2

% mean difference = $\{(x1-x2)/(x1+x2)/2\}$ expressed as a percentage

Table 8.7. Comparison of Correlations from Previous Studies

	Age	Chol	Trig	HDL	ApoB	ApoA1	Smoke	BMI
1.Extent ⁵⁹³	+	+	+	-	+	-	-	-
Severity	-	-	+	+	+	-	-	-
2.Extent ^{207*}	+	+	-	+	+	-	-	-
Severity	-	-	-	-	-	-	-	-
3.Jenkins ⁵⁹¹	+	+#	-				+#^	-
206	-	+	-	-	+	-		-
601	+	+	+	+				
4.Severity ⁶⁰⁰	+	+	+	+				
5.Severity ⁶⁰²	+	-	-	-			+	-
6.Present study								
extent*	+	+	-	-	+	-	+^	-
severity*	+	-	-	-	+	-	+/-	-
Jenkins	+	+/-	-	-	+	-	+^	-

* = same scoring system, # = age < 55 years, ^ = packyears, + = positive, - = negative, and +/- = borderline relationship. Blank indicates assessment not evaluated. Extent = extent of disease according to the investigator. Severity = severity of disease according to investigator. Jenkins = Jenkins severity score.⁵⁹¹

Table 8.8. Variables correlating with the CAD scores.

ANGIO SCORE	PLATELET VARIABLE	r	LIPID VARIABLE	r	OTHER VARIABLES	r
Normseg	SERTXB2	0.24*	CHOL	-0.38#	AGE	-0.23*
	MPV	-0.24*	APOB	-0.38#	PACKYR	-0.44#
	PRPPC	0.34#	C14:0	-0.35#	Ho	-0.31*
			C18:1w9	0.30*		
CAS			CHOL	0.29*	HbA1C	0.31*
			APOB	0.35#	SST	0.38**
			C14:0	0.32*	SBP	0.35#
			C20:4w6	-0.24*	DBP	0.30*
					AGE	0.27*
mCAS			CHOL	0.33#	HbA1C	0.30*
			APOB	0.35#	SST	0.38**
			C14:0	0.32#	SBP	0.30*
					DBP	0.27*
					AGE	0.29*
					PACKYR	0.31*
CSS	SERTXB2	-0.24*	C14:0	0.34#	HbA1C	0.27*
	MPV	0.27*			AGE	0.25*
	PRPPC	-0.31*			Ho	0.35*
					NYHAFC	0.31*
mCSS	LT50ADR	-0.28*	C14:0	0.36#	HbA1C	0.25*
	MPV	0.26*			AGE	0.25*
					PACKYR	0.31*
					Ho	0.35#
					NYHAFC	0.34#

Table 8.9. Variables correlating with the CAD scores.

ANGIO SCORE	PLATELET VARIABLE	r	LIPID VARIABLE	r	OTHER VARIABLES	r
LES100	SERTXB2	-0.26*	C14:0	0.26*	Hb	-0.29*
					SBP	0.25*
					Ho	0.41#
					NYHAFC	0.40**
LES90/99	MPV	0.25*	C14:0	0.24*	AGE	0.34#
			C20:2w6	-0.26*	HbA1C	0.27*
			C22:5w3	-0.25*	BSL	0.33*
					FIBRIN	0.26*
LES75/90			C14:0	0.25*	PACKYR	0.31*
			C16:0	0.24*		
			C22:5w3	-0.29*		
LES50/75	LT50ADR	-0.36#	CHOL	0.36#	AGE	0.31*
	SERTXB2	-0.29*	TRIG	-0.26*		
			APOB	0.30*		
			C14:0	0.24*		
LES25/50			CHOL	0.30*	Hb	0.35#
			APOB	0.33#	Hc	0.39#
					HbA1C	0.42**
					SST	0.52
					SBP	0.31*
					DBP	0.35#
					Ho	0.32*
LES10/25			CHOL	0.37#	SBP	0.25*
			APOB	0.36#	DBP	0.29*
JENKINS	LT50ADR	-0.31*	APOB	0.27*	HbA1C	0.25*
	MPV	0.24*	C14:0	0.32#	AGE	0.28*
			PACKYR		0.29*	
LVS	LT50ADR	-0.30*			No MI	0.43##
	PRPPC	-0.27*				
CS	SERTXB2	-0.24*			NYHAFC	0.39**
					Ho	0.30*

Table 8.10. Multivariate analysis with the CAD scores as the dependent variable.

Dependent Variable	Independent Variable	B	SE B	Beta	R sq	P value
NORMSEG	APOB	-0.142	0.062	-0.537	0.288	0.039
	C14:0	-0.012	0.005	-0.472	0.508	0.014
(Other variables entered were clotted whole blood TXB2 level, MPV, DOB, cigarette packyear and the MMPI hostility score)						
CAS	SSFT	0.062	0.030	0.357	0.128	0.045
	C14:0	0.085	0.030	0.487	0.261	0.012
mCAS	SSFT	0.051	0.025	0.361	0.251	0.043
	C14:0	0.069	0.024	0.483	0.251	0.015
(Other variables entered were apoprotein B and C20:4w6)						
CSS	LT50ADR	-31.089	6.770	-0.824	0.678	0.001
mCSS	LT50ADR	-30.589	6.1397	-0.844	0.713	0.006
(Other variables entered were TXB2 in clotted whole blood, MPV, C14:0, DOB, cigarette packyears and the MMPI hostility score).						
JENKINS	LT50ADR	-12.419	3.908	-0.647	0.419	0.007
	MPV	0.350	0.150	0.415	0.590	0.003
(Other variables entered were APOB, C14:0, DOB and packyears of cigarettes smoked).						
LVS	No. AMI	1.198	0.557	0.333	0.111	0.038
	LT50ADR	1.248	0.534	0.347	0.208	0.015
(Another variable entered was the CSS which had no effect on the relationship)						

CHAPTER 9

GENERAL DISCUSSION

9.1 SUMMARY

The framework of this thesis is that of an observational study with a cross-sectional survey of two homogeneous groups which differ in relation to the outcome of interest, namely CHD. Specific variables under evaluation were measured in both study groups and compared for any differences. The study also provided a comprehensive examination of the relationship between the specific measures under evaluation (platelet function) and various other characteristics that may have a potential influence on the relationship between the measures being examined and the outcome of interest.

The study populations were carefully defined and the limitation of major extraneous confounding factors was an important goal. Individuals in each of these populations were examined by means of standardised techniques, a common protocol and the same interviewer.

The observations presented in this Thesis provide evidence that males with premature CHD have platelets which are more reactive. Criteria of increased platelet reactivity as well as platelet volume were significantly greater in males with angiographically proven and stable CHD. Furthermore, measures of this reactivity were related to the severity and number of obstructive coronary artery lesions. In contrast, a lipoprotein measure, namely apoprotein B, was more predictive of the extent of arterial involvement by CAD as defined angiographically. These observations provide supportive evidence for the hypothesis that the development of CHD in young males relates to a combination of thrombogenic and atherogenic risk factors. The pathophysiological mechanisms involved in this increased platelet reactivity cannot be determined from this study given the cross-sectional observational design with retrospective ascertainment of cases.

9.2 CAUSATION AND CHRONIC DISEASE

9.2.1 Relative Importance of CHD Risk Factors

Undoubtedly men with premature CHD have abnormalities of plasma lipids, LDL and total cholesterol, apoprotein B and A1, and lipoprotein (a),^{987,988} and also HDL cholesterol and triglyceride levels.^{988,989} The relative contribution of these to the risk of CHD is uncertain, although apoprotein A1 and B levels may be better predictors of premature CHD than other plasma lipids.⁶⁰⁷ Because of the diverse nature, and more importantly, the non-specific predictive value of these lipid risk factors, much effort is now being employed to improve the precision of prediction of risk from more refined lipoprotein profiles,⁹⁹⁰ profiles which may more accurately reflect the basic mechanisms that involve lipids in atherogenesis. Unfortunately, the mechanisms have not been accurately determined, and, until they are, the likelihood of providing accurate predictive measures remains low. Our results are consistent with the observations for those lipoproteins measured, and also suffer from the same problem of poor sensitivity and specificity in the study groups.

The question as to which of the coronary risk factors are important is a question of

great practical relevance, both for the clinician and for public health.⁹⁹¹ The current simplicity in measurement of risk may be incorrect. Identification of risk factors for each stage in the development of CHD would be a more productive approach.²⁰² *Identification of a combination of different pathophysiological risk factors, that is, risk for atherosclerosis co-existing with risk for thrombosis, may indeed provide a better approach for improving our precision of CHD risk assessment.*

Just as the lipoprotein abnormalities contributing to risk have been difficult to delineate concisely, so to has the identification of thrombogenic risk factors. Many potential mechanisms exist which provide a basis for increased thrombogenic risk. Besides the presence of platelet function changes, other potential thrombogenic risk factors include increased blood viscosity,^{63,64} impaired fibrinolysis,^{40,41,60,316} an increased fibrinogen level,^{60,61} increased coagulation factors, particularly factor VII,⁶⁰ and elevated arterial wall shear stress.⁹⁹² The interrelationship of these factors, and even the co-existing influence of abnormal lipoproteins on these factors⁶⁴ provide a "tangled web" of risk factors possibly greater than previously proposed.⁶³⁵

The results of this present study show that platelet function appears to be more important for the development of severe lesion development and lipids are more relevant to lesions with mural involvement. Whether the latter is a prerequisite for the former cannot be determined from this observational study. Nevertheless, the association of platelet aggregability and the severity of angiographically defined CAD still holds when the other measured risk factors are held constant.

9.2.2 Proving Causality

The epidemiological evidence that increased platelet aggregability is not only a risk factor but a causative risk factor is increasing. The need for an ability to predict thrombotic risk has previously been reviewed and emphasised.^{32,54,475,835,878} There is a large body of data supporting such a requirement, and the observations in this Thesis add to and emphasise the emerging body of knowledge in this area. However, any examination of the evidence for this claim requires the application of those criteria for asserting causality which were outlined by Sir Austin Bradford Hill,⁹⁹³ and amplified by others.⁷³⁷

In studies of chronic diseases, such as atherosclerosis and CHD, the clinical discovery of the disease is often years or decades after the biologic onset, and the time of this onset is unknown.⁹⁹⁴ Therefore, information necessary to measure the prevalence, incidence and duration of the particular disease is not always available, making it difficult to determine causative factors for the disease. Not surprisingly, the criteria for inferring causation of disease by any specific attribute or agent has long concerned clinical epidemiologists. The requirement for procedural and inferential methods had been stimulated by the now well recognised associations between specific measurable attributes of smoking⁹⁹⁵ and blood cholesterol,^{996,997} and chronic disease occurrence in populations and individuals. However, the problem of multiple causation of disease, and the specificity of proposed causative factors for causing disease, have provided difficulties for the epidemiological investigation of many chronic diseases.^{998,999} Furthermore, any causative attribute of low biological pathogenicity may only produce clinical disease with exposure at a given age, if resistance

is impaired, or in the presence of a pathogenic or nonpathogenic cofactor.⁷³⁷ For example, Lilienfeld⁹⁹⁹ has highlighted the concept of "vectors" occurring in chronic disease, as well as in infectious diseases. This consideration is one form in which an identified and measurable attribute can be a marker for the true causative factor, and is associated with the disease via this secondary dependence.

There are further intuitively apparent limitations in the pursuit of causality.^{471,737} The same pathological or clinical state can be produced by different aetiological agents. Such agents may be different depending on geographic area, age group, and varying patterns of host susceptibility. Indeed, some diseases require the presence of two or more agents or cofactors acting to produce the disease state. Equally, a single agent may produce different clinical and pathological responses in different settings. The nature and severity of the host response following exposure to an agent varies with specific host characteristics. These include behavioural patterns, socio-economic level, age, immunological states, and pathogenic or non-pathogenic cofactors.⁷³⁷ As well, the interaction of the environment and genetic constitution is often, if not always, of importance.

These considerations invariably lead to numerous problems in the design of cause-effect research, giving rise to measurable and immeasurable bias.⁴⁷¹ Experimentation and the determination of biological mechanisms provide the most direct evidence of a causal relationship. Unfortunately, this approach to the understanding of the pathogenesis of chronic disease cannot always be implemented, and much depends on biological inferences from epidemiological studies of such diseases. Thus, the question arises, when observation reveals an association between variables and disease, what aspects of that association should be considered before concluding the presence or absence of a causative association. A series of requirements had been proposed when their necessity for the investigation of chronic disease became evident.⁹⁹⁸ This epidemiological approach expanded with time. Sir Austin Bradford Hill proposed more rigorous criteria, with all of which "*we should study association before we cry causation*".⁹⁹³

A unified concept for a set of criteria for inferring causation has now been well established in the epidemiological literature.^{470,471,737,993} Sir Austin Bradford Hill originally proposed 9 *criteria for consideration when evaluating whether or not an association between a risk factor and disease is causal*.⁷³⁷ These criteria have been defined and presented in detail by Evans⁷³⁷ as:

1. Prevalence of disease should be significantly higher in those exposed to the putative cause, than in case controls not so exposed.
2. Exposure to the putative cause should be present more commonly in those with the disease than in controls without disease, when all risk factors are held constant.
3. The incidence of the disease should be significantly higher in those exposed to the putative cause than in those not so exposed as shown in prospective studies.
4. Temporally the disease should follow exposure to the putative agent with a distribution of incubation periods on a bell shaped curve.
5. A spectrum of host responses should follow exposure to the putative

cause along with a logical biological gradient from mild to severe.

6. A measurable host response following exposure to the putative cause should regularly appear in those lacking this before exposure (e.g. antibody, cancer cells), or should increase in magnitude if present before exposure; this pattern should not occur in those not so exposed.

7. Experimental reproduction of disease should occur in higher incidence in animals or man appropriately exposed to the putative cause than in those not so exposed; this exposure may be deliberate in volunteers, experimentally induced in the laboratory, or demonstrated in a controlled regulation of natural exposure.

8. Elimination or modification of the putative cause or, of the vector carrying it, should decrease the incidence of the disease (e.g. control of polluted water or smoke, or removal of a specific agent).

9. Prevention or modification of the host's response on exposure to the putative cause should decrease or eliminate the disease (e.g. immunization, drugs to lower cholesterol, etc.).

10. The whole proposal should make biological and epidemiological sense.

Therefore, the position of increased platelet aggregability as a risk factor must satisfy the criteria outlined if a causal relationship is to be substantiated from an epidemiological perspective.

9.2.3 Prevalence of Disease

The possibility of chronically abnormal platelet function being a risk factor for CHD has been a subject of evaluation for many years.^{52,55,94,166,624,766,848,851,906,1000,1001} For example, younger males with CHD having a normal coronary angiogram or single obstructive arterial lesions have platelet hyperaggregability.⁵²

9.2.4 Exposure to Potentially Causal Factors

The results of this Thesis show that, as a group, males with premature CHD have a measurably increased platelet reactivity in comparison to males currently without CHD. That is, prevalent cases of males with CHD have evident a greater exposure to the putative causal factor, platelet hyperreactivity, than those without CHD.

9.2.5 Coronary Heart Disease Prognosis and Platelet Function

Two recently reported prospective studies provide further support for increased platelet aggregability as a risk factor for CHD.^{901,902} Both studies indicate that non-fatal MI and fatal CHD cases may have similar platelet abnormalities.⁴⁷⁵ Moreover, prognosis after non-fatal MI is significantly worse in patients with high spontaneous platelet aggregation.⁵⁹ A previous case-control study also presents a prognostic association between platelet aggregation and vascular disease.⁸⁵¹ The relationship between severity of obstructive coronary arterial lesions and the aggregability of platelets in response to adrenaline adds a cohesive dimension to these observations linking platelet aggregation and prognosis. Which platelet factor(s) best predict increased risk for MI require elucidation.⁴⁷⁵ At present the relevant *in vitro* platelet function test which simulates the pathological events of coronary thrombosis is unknown.⁹¹ Nevertheless, further criteria for establishing causality have been pro-

vided by these studies. Exposure to the putative cause, that is, increased platelet aggregability,^{901,902} and the subsequent incidence of disease are prospectively related.⁹⁰² Moreover, temporally, the acute coronary events occur after documentation of the platelet hyper-reactivity.

9.2.6 "Host" Response and Biological Gradient of Disease

If a true relationship exists between discrete obstructive lesions in angiographically defined CAD and increased platelet reactivity, and similarly between extent of lesions (but not severity) and a lipoprotein measure, one could expect such observations to be reflected in the pathology of atherosclerotic lesions in the young. Human atherosclerotic lesions differ in the nature and quantities of cells and interstitial components of which they are formed.^{1002,1003} Clearly, factors other than the lipid component and the incorporation of thrombus into an atherosclerotic lesion are also clearly involved in the formation of the various parts of atherosclerosis, for example, cell necrosis.¹⁰⁰³ The process of necrosis possibly involves modified forms of LDL, in particular oxidised LDL which is cytotoxic.^{131,260,1003} Nevertheless, an evaluation of the pathology of lesions over time does provide support for the differential association of lipoproteins with early lesion development and thrombosis with the development of obstructive lesions.

An alteration in the lipid constituents of atherosclerosis occurs with aging, from lipid-laden macrophages in pre-puberty, to lipid droplets in smooth muscle cells by puberty, and to the beginning of accumulating extra-cellular lipid after puberty.¹⁰⁰² In the third and fourth decade of life fibroatheroma develop. Importantly, at this later stage, collagenisation and thickening of atheroma are more marked when evidence of thrombotic deposits are present on the surface or within lesions.¹⁰⁰² Moreover, endothelium over established human plaques often shows focal denudation injury with adhesion of a platelet monolayer.⁷³³ In deep intimal injury, thrombus may form within plaque altering the configuration and expanding the volume, providing a cause of episodic and rapid plaque growth.⁷³³ Thrombus formation, or organisation, and acute or subacute growth of atherosclerotic plaques commonly occur together.^{40,733}

Therefore, both the current understanding of lesion development and our angiographic observations are consistent. Moreover, there is a biological gradient of association between lesion severity, the host response, and one putative cause, increased platelet reactivity.

9.2.7 Platelet Function and Coronary Thrombosis

A latent but increased potential for platelet aggregation may contribute to the development of CHD because of increased thrombus propagation resulting in coronary occlusion and/or acute lesion expansion. Alternatively, long-term diffuse atherosclerotic disease progression may be increased because of platelet adherence or aggregation on vascular endothelium. Finally, a combination of enhanced coronary thrombosis and the potential for acute plaque expansion may both contribute. The latter proposal is supported by the pathological studies described above, as well as the relationship between heightened platelet reactivity and stenotic lesions observed

in our study. In addition, clinical observations regarding the time dependent effect of chronic platelet inhibition with low-dose aspirin on CHD favours platelets being involved in acute lesion and thrombus formation.⁶⁹⁵ The reduction by aspirin of the cumulative incidence and cumulative relative risk for AMI occurs soon after initiation of therapy and does not change over time.⁶⁹⁵ There is also no treatment effect for the development of angina pectoris.^{695,697} This concept, however, relies on the assumption that angina is a clinical measure for the outcome of atherosclerosis progression, and this may not be reliable. Furthermore, the follow-up duration of 5 years may not be sufficient to affect the process.⁶⁹⁵ Low-dose aspirin also reduces the risk of subsequent AMI in patients with established clinical CHD, manifest as asymptomatic, and less so with symptomatic myocardial ischaemia.¹⁰⁰⁴ The results of the above studies are consistent with the association of platelet aggregation with the development of discrete obstructive lesions and hence disease severity.

Clearly evidence exists that modification of one possible contributing factor, namely platelet aggregation, with aspirin, reduces the incidence of one clinical expression of severe CAD, namely AMI. It is speculative to propose that the clinical relationship can be used as an indicator of the underlying process. However, the preliminary results of a trial assessing the effect of aspirin on atherosclerosis defined by quantitative coronary angiography suggest that aspirin therapy may reduce new lesion formation but not intramural atherosclerotic progression.¹⁰⁰⁵ Such a finding is very consistent with our results of an association between obstructive lesions and platelet aggregation, and also with the clinical data on the prevention of AMI but not angina pectoris by aspirin in the Physicians' Health Study.⁶⁹⁵ In addition, increased platelet aggregation in the morning, and upon rising, may contribute to the increased occurrence of AMI at this time.^{224,903,1006,1007} Aspirin reduces the incidence of AMI in the morning.¹⁰⁰⁷

9.2.8 Therapeutic Efficacy of Reducing Platelet Aggregability

The above studies only provide tentative evidence that the incidence of the disease is altered by anti-platelet agents, with a reduction in coronary thrombus formation and consequently a reduction in severe obstructive lesions. Nevertheless, the response of the "host" in terms of the clinical expression of disease is clearly altered, with a marked reduction of CHD events.^{695,1004,1007} Effectiveness of treatment was established before the accumulation of evidence that platelet aggregability may be a risk factor for CHD.^{696,698,992,1008-1010} Therefore, in retrospect, the inhibition of platelet aggregation could be interpreted as the treatment of a risk factor, both for the primary and the secondary prevention of CHD, in the same way as attempts are made to alter lipid metabolism, mainly by lowering blood cholesterol. Considering the success of aspirin in the clinical trials showing a reduction in cardiovascular mortality for both those without known preexisting disease and those with established disease, the importance of arterial thrombosis in the clinical expression and prognosis of CHD is undeniable. In light of the therapeutic efficacy of aspirin, the presence of thrombogenic risk factor(s), particularly involving platelet functions, therefore should not come as a surprise. Nevertheless, the thrombogenicity of an ulcerated or fissured atherosclerotic plaque may be sufficient without an added need for increased platelet aggregability.³²

9.2.9 Biological Plausibility and Experimental Reproduction

The biological plausibility and laboratory evidence in support of the possibility of platelet hyperaggregability being a risk factor for CHD and also other forms of vascular disease has been reviewed previously^{32,40-42,733} and are summarised in the introduction Chapter of this Thesis.

9.2.10 Summary

The results presented in this Thesis contribute to the criteria required to establish causality of a factor in epidemiological terms. Firstly, heightened platelet activation is present more often and to a greater degree in individuals with CHD than in a control group without CHD. Secondly, there is a biological gradient in the predicted disease outcome, namely severity and number of discrete obstructive coronary artery lesions and the aggregability of platelets to adrenaline. The overall body of literature currently provides a strong argument that a potential for increased platelet aggregation is a causative risk factor for the clinical expression of CAD, and is manifested as the thrombotic complications of this disorder.

9.3 QUALIFICATIONS INFLUENCING THE CONCLUSIONS

9.3.1 Major Limitations

Because of the inherent bias in case-control studies and the very real potential for unmeasured and unknown confounding factors, it is necessary to state the main limitations of the study.

1. All results reported in this thesis represent associations which do not necessarily identify an antecedent or consequent factor.
2. The study variables were based on a single measurement on each individual and therefore only approximate the true value for each characteristic measured.
3. The study did not directly control the behaviour of the individuals being examined and depended on voluntary cooperation undertaken in good faith.
4. The study design did attempt to distinguish areas of potential bias in order to limit confounding factors, but relied heavily on statistical analysis for the adjustment of variables concomitant with, or extraneous to, the measurements of interest.
5. Much of the analysis of the study was multivariate, and therefore Type I statistical errors are more likely. This is a commonly known limitation of studies in which large numbers of statistical tests are performed. Many of the observations, therefore, are only suitable for gathering potential associations requiring further investigation rather than being inference forming or hypothesis testing.
6. The study populations were carefully defined and hence the observations cannot be generalised to the entire population of CHD patients.

9.4 STATISTICAL METHODS AND MULTIVARIATE ANALYSIS IN CASE-CONTROL STUDIES

Given the major limitations described above, in particular points 4 and 5 which partly relate to the use of multivariate analysis, further discussion of the appropriateness of the statistical methods utilised is of importance. The limitations of case-control studies are well recognised,^{370,471,473,1011,1012} and mandate a concise understanding of the limitations of observations arising from studies using such methodology.

9.4.1 Specific Limitations of Case-control Studies using Coronary Angiography for Assessment

Certain areas of bias exist in case-control studies which are unavoidable, as discussed in Chapter 4. Furthermore, important limitations to the use of angiographic assessment of CAD are well recognised and discussed in Chapter 8. When the use of coronary angiography is part of the case-control design, appropriate caution has been urged.⁴⁷⁴ Knowledge is often gained not through a single study but rather through several different and consistent sources of information which helps to ensure that the conclusions from one study are not peculiar to that study.¹⁰¹³

The discussion following, however, will aim to illustrate how some of these *inherent limitations are more likely to lead to a false negative outcome in this study by decreasing the true differences and associations between the study groups*. Moreover, the statistical analysis within this study has followed the recommendation that more than one approach should be undertaken in the analysis of a single study to also ensure that the results are not an artifact of the study design or statistical methods.¹⁰¹³

9.4.1.1 Retrospective case selection. The retrospective design of a case-control study leads to a study beginning with a potentially unrepresentative group of cases.^{471,1014} The diagnosis is made prior to the selection and can lead to a prevalence-incidence bias.⁴⁷¹ The case group in this study represents cases of non-fatal CHD. Also, it is possible that those with less severe disease and/or left ventricular dysfunction are underrepresented. Cases of sudden death, large MIs and left ventricular dysfunction, unstable ischaemia following MI, ischaemic induced sudden death and/or recurrent MI will more likely not be selected. Severity of CAD, as measured by the number of lesions and their degree of encroachment into the lumen are related to prognosis of CHD and the aforementioned factors. Given that increased platelet aggregation is associated with increased cardiovascular death and with an adverse prognosis,^{901,902} if such groups were absent from the case group because of premature death, the likelihood of finding a difference in platelet function would in theory be reduced, diluting the results of this study. Since cases of nonfatal CAD undergoing coronary angiography appear to be representative of all non-fatal CHD cases in a population,^{474,1011} then our results demonstrate that not only are those with an increased incidence of cardiac events, including cardiac death, associated with increased platelet aggregation,^{901,902} but also cases of non-fatal and clinically stable CHD.

Moreover, CHD risk factor levels are more likely to have been altered compared to the pre-morbid levels. The risk factors may be influenced by the physical damage associated with a MI, by the medical or surgical treatment of the disease and by lifestyle changes that have been strongly encouraged. Indeed, this has happened in the case group as illustrated by a decrease in the total blood cholesterol level over time. Such changes due to medical treatment and lifestyle adjustments are more likely to decrease the potential for determining risk factor differences between the groups. Nevertheless, this important area of bias may also be in the opposite direction. This emphasises the fact that case-control studies are useful mainly as initial screening studies in order to determine areas of potential importance requiring more detailed investigation.

9.4.1.2 Coronary angiography as an assessment method. The major problem with case-control studies, as discussed above, is the various forms of selection bias. Such bias may be further enhanced by using angiographic methods of assessment.⁴⁷⁴ For example, an elevated cholesterol level may lead to a selection bias for the performance of angiography.⁴⁷⁴ *The case group in this study, however, was not selected on the grounds of the angiography but because they had presented with clinical CHD, most having had a MI.*

Combining cases with and without a prior MI may also lead to error concerning which risk factors are related to atherosclerosis and which lead to the precipitation of the clinical event.⁴⁷⁴ However, the use of angiography in case-control studies allows the examination of risk factors specifically associated with MI independent of atherosclerosis¹⁰¹¹ and with different angiographically defined morphology of CAD.²⁰⁷ Moreover, the measures can be used to strengthen the evidence for a dose-response relation between risk factors and coronary artery atherosclerosis.¹⁰¹¹

9.4.1.3 Control group selection. Because the control group did not undergo coronary angiography, some individuals in this comparison group may have subclinical CAD. Such a misclassification of subjects by disease status, that is CAD although not CHD, could dilute the degree and/or type of differences or associations found, giving a higher possibility of false negative associations for risk factors for the development of atherosclerosis. However, such a control group selection bias is less likely to affect the risk factor difference(s) (e.g. thrombogenic risk factors) that may transform underlying atherosclerotic CAD into the clinical expression of CHD. Interestingly, and of particular relevance to our observations, multivariate risk factor models using the classic major risk factors, are similar when using either a control group from the general population without symptoms, and those with symptoms but angiographically definable normal coronary arteries.¹⁰¹¹ The selection of the controls as a group without clinically expressed disease was undertaken with the knowledge that asymptomatic atherosclerosis is very prevalent in Western societies.^{38,639} Of equal importance is the fact that AMI can occur in individuals with minimal angiographically defined CAD.¹⁰¹⁵

Given the above observations, the argument could be made that the method of *selection of controls and cases employed in this study enhanced the chance of eliciting factors that can transform atherosclerosis, which is so prevalent in the asymptomatic form even at the age of our study groups^{38,639} into the clinically expressed condition.*

9.4.2 Specific Limitations with Multivariate Regression Analysis

In the initial attempt to understand a possible interaction between variables, it is reasonable to follow an exploratory and descriptive approach rather than a strictly inferential one.¹⁰¹³ Indeed, as stated earlier, this was the main purpose of the current study. Nevertheless, when a dependent variable is used as a focal point in an analysis by one of the forms of multivariate analysis, such as multiple regression analysis, it is possible to validate the results because they produce a prediction about the dependent variable used as the focus.⁴⁰⁴ Furthermore, appropriate methodology must be employed to reduce the potential violations of the assumptions made when using multivariate regression analysis.^{471,1016}

9.4.2.1 Validation of Associations from Multivariate Analysis. The predictions arising from multivariate regression analysis can be validated by a number of processes,⁴⁰⁴ for example:

(a) *External validation* by evaluating the same analysis in another group, in one or more control groups, or compare the prediction with previous observations in the literature.⁴⁰⁴ As demonstrated in Chapter 5, the multivariate reduction of variables by multivariate regression used to evaluate the relationship of lipoproteins and CAD was in general concordant with the literature.

(b) *Validation internally*, that is within the study design, by demonstrating expected findings within other variables in the study.⁴⁰⁴ Again, such internal validation has been demonstrated and discussed in Chapter 5. For example, the predictable and persistent relationship between age and the angiographic scores is an expected observation.

(c) Demonstrable by *use of different statistical methods to show that the results are not an artifact of that model*.¹⁰¹³ Categorisation of the independent variables with significant relationships with the angiographic scores and subsequent analysis of variance of the mean angiographic scores of these groups demonstrated confirmatory results indicating that the correlation and multivariate observations were not due to chance or an artifact of the model used.

9.4.2.2 Number of variables in stepwise regression analysis. When many variables are being examined in stepwise regression analysis, such methods can find significant factors when no real association with the dependent variable exists. In general, if there are n observations for the least frequent category of a binary response variable, you should not examine more than $n/10$ variables in order to derive a model which is somewhat reliable.¹⁰¹⁶ Therefore, in this study attempts were made to limit the number of independent variables entered into the regression model to 6 or less.

9.4.2.3 Regression dilution bias. Overadjusting in multivariate analysis can lead to false negative results due to the effect of regression dilution bias. This arises for a number of reasons. Firstly, if a variable is closer along the pathogenic pathway but is not recognised, the factor under examination may not achieve significance and both are ignored. Secondly, the effects of collinearity are a well known source for false positive associations when the true related variable to the dependent variable is not included in the regression model. In addition, entering both of the related varia-

bles may result in a dilution of the predictive strength of the true associated variable. Finally, the presence of very precisely measured variables with less or little predictive value for the dependent variable may be given more significance than a more closely related factor which is less able to be precisely measured.

Such problems are often difficult to discern in multivariate analysis. Their prevention requires an understanding of the basic model being used, an intimate knowledge of the independent variables and their interrelationships, and of course the disease or dependent variable itself. Thus a large degree of clinical and medical scientific acumen is often required in this regard. Even then, inappropriate variables will be given a predictive association as well as a degree of false negatives from the various forms of dilution bias. Therefore, the importance of comparison to other studies and replication of results becomes critical. Indeed, knowledge is often gained not through a single study but rather through several different and consistent sources of information which helps to ensure that the conclusions from one study are not peculiar to that study.¹⁰¹³

9.4.3 Within Person Variability

There are well recognised sources of variation in measurement that affect comparisons made in both clinical and epidemiological studies. This study is concerned with ascertaining and describing certain variables in a predefined case group and a control group. Comparison of the variables of interest and inferences made from the surveyed data were used to support or negate a proposed hypothesis. Sources of variation in measurement affect any comparisons to be made quite separately from any effect arising from the sample of the population under study. Such possible sources of variation include inter-observer variability, observer-patient interactions and intra-individual variability.⁵⁴⁹ These possible sources of error emphasise the importance of standardisation of study procedures and methodology, as far as possible. The effect of within-person variability in measurements leads to the possible misclassification of disease, or the bias introduced by the tendency of regression to the mean of those variables measured.

Regression to the mean refers to the phenomenon that an extreme variable on its first measurement will tend to be closer to the centre of the distribution on later measurements. This variability occurs because of variation in the biological parameter being measured, as well as the variation in the measurement itself.⁵⁵⁰ Hence, it arises not only because of within-person variability, but also inter-observer error and observer-patient interactions. This is of particular importance for studies wherever the sample is chosen because of the measurement of a biological variable above or below a specified level. Even the prospective collection of data is subject to numerous errors which include the effect of regression to the mean and subsequent misclassification of persons by disease status. These errors could lead to true relationships being obscured or attenuated, and in intervention studies, improvement in disease status being falsely attributed to treatment.

This centripetal movement of data particularly affects studies which recruit their sample after a screening process has demonstrated their above or below normal value. Some of these values may be due to random fluctuation of within-person or

measurement variations, and, as discussed above, will return towards normal levels in subsequent measurements, unassociated with any real changes within the individual. This does not apply when the subjects are chosen randomly, since a random sample would probably contain a full range of the possible variables available. Moreover, if all subjects are selected in a given period or if the selection criteria do not involve a single biological measurement(s), as in this study, the problems associated with regression to the mean do not arise. In addition, the utilisation of a control group having the same measures performed with the same methodology reduces the problem.⁵⁴⁹

9.4.4 Hidden Bias

It is unlikely that all relevant variables have been identified and measured in this observational study, a fact that is inherent to the design and always a potential area of bias in case-control studies.¹⁰¹² Some relevant variables may have been excluded from the analysis even though they have been measured. Other variables may have been included even though they in reality are not important determinants of the dependent variable measured. The observations therefore must be interpreted with this in mind as one can only use the available data specific to a study. Other relevant measures include lipoprotein (a),⁶⁹ oxidised lipoproteins,²⁶⁰ postprandial lipids,¹⁰¹⁷ factor VII,⁶⁰ plasminogen activating factor inhibitor,³² other haemostatic variables⁵⁶ and insulin resistance¹⁰¹⁸ to name but a few of the legion of potential factors.²³⁵

9.5 FINAL CONCLUSIONS

9.5.1 Platelet Function in CHD Patients With and Without Myocardial Ischaemia on Exercise Testing

Short-term maximally strenuous exertion may cause *in vitro* activation of platelets, as measured by an increase in plasma BTG, irrespective of the presence or absence of ischaemia. However, exercise-induced myocardial ischaemia *per se* does not appear to activate circulating platelets, nor are previously activated circulating platelets associated with exertional ischaemia as assessed by the methods in this study. Patients with exertional ischaemia may produce less serum TXB2 at rest than those without evidence of ischaemia. Those with exertional ischaemia group have an increased production of TXB2 in clotted whole blood following exertion, whereas those without ischaemia do not.

9.5.2 Effect of the Combination of Cardiovascular Drugs, Metoprolol and Nifedipine, on Platelet Function in Patients with and without Myocardial Ischaemia Produced on Exercise Testing

There is no evidence of activated circulating platelets following exertional ischaemia and no measurable effect of the drug combination of metoprolol and nifedipine on *in vivo* measures of platelet activity. However, an increase in prostaglandin biosynthesis *ex vivo* in whole blood, as measured by TXB2, is associated with exercise induced myocardial ischaemia and is suppressed by a combination of a beta-blocker and a calcium entry blocker. The drugs may produce their effect by reducing myo-

cardial ischaemia, BP or, alternatively, by directly decreasing blood cell prostaglandin formation. In addition, it may be possible that a reduction of whole blood prostaglandin activity during thrombus formation in individuals with myocardial ischaemia may contribute to the protective effect of these drugs against acute, and transient obstructive thrombotic coronary episodes.

9.5.3 Blood Cells and the Production of TXB2 in Clotting Whole Blood

The amount of TXB2 produced in clotting whole blood is influenced by both platelet specific factors and environmental factors in the circulation, both molecular and cellular, and these differ in patients with CHD and controls without CHD. Specifically, there is a relationship between serum TXB2 levels and platelet morphology (MPV) as well as blood cell indices in the young CHD group. In patients with stable CHD who have no evidence of measurable *in vivo* platelet activation, serum TXB2 is not related to measures of *in vivo* platelet function. In contrast, in the control group serum TXB2 was related to the degree of platelet reactivity as indicated by the stronger relationship with *in vitro* platelet aggregation, the PCR and plasma BTG levels.

The observations provide further evidence for potential variation in the ability of the platelet to produce TXB2 levels in different disease states.

9.5.4 Association Between Blood Lipids and Angiographically Defined CAD

In males with premature CHD, lipoprotein abnormalities are associated with atheromatous involvement of the arterial wall, and not with severe lesions encroaching into the lumen. The development of generalised atherosclerosis may merely be a contributing factor to the clinical expression of CAD in the form of CHD. The formation of acute severe stenoses may have different risk factors and mechanisms than that for the development of diffuse atherosclerosis.

9.5.5 Lipoproteins and Platelet Function

There exists a large but unclear body of evidence implicating a relationship between lipoproteins and abnormal platelet function. Consistency in the literature with this study adds support for an influence of the blood cholesterol level on adrenaline-induced platelet aggregation. However, cholesterol may be a surrogate measure for another factor(s) which may have an aetiological relationship with abnormal platelet function, such as oxidised LDL.

9.5.6 Dietary Differences Between the Study Groups

The subjects in the case group, on the average, appear to eat less fatty foods, less dairy products and more fruit than the control group. There was also a reduction in the contemporary cholesterol level compared to previous levels in the same group, suggesting a change in habitual diet, occurred following the diagnosis. The influence of these dietary differences could have led to reduced platelet reactivity and measures of aggregation in the case group compared to the control group. However, it cannot be excluded that a change in diet, once the previous cholesterol level was

known, may have lead to enhanced platelet reactivity due to changes in dietary fatty acids. For example, an increase in polyunsaturated fatty acids, which are susceptible to oxidation, without an appropriate level of antioxidants within the body or within lipoprotein molecules such as LDL, may lead to the formation of oxidised LDL more easily. Oxidised LDL may then have an adverse influence on platelet function, making them more susceptible to aggregation, and thus leading to the differences demonstrated in this study. Unfortunately, such a proposition cannot be discounted in this observational study.

9.5.7 Platelet Function Differences Between Males With and Without CHD

The MPV in young males with CHD was greater than in normal control subjects and the increase was present more than 3 months after an infarct. The higher MPV occurred without evidence of ongoing *in vivo* platelet activation. The MPV is therefore an identifiable risk factor in young males with established CHD supporting similar conclusions made by others. That is, there is a statistically significant difference between a group with disease compared to a group without disease, without there being grounds for inferring causality. As with most risk factors, the large overlap between individuals in both groups prevents the measurement of the MPV from being a clinical useful parameter for disease identification or assessment of severity, at present. This was found to be a feature of all those platelet function measures which differed between the two groups.

Other interacting influences, namely the content of plasma fatty acid, may have resulted in the difference observed. Hence the increased MPV may be only a marker for another pathophysiologically abnormality present to a greater degree in the CHD group.

A difference in the PCR between the control and case groups was also demonstrated, the PCR being lower in young males with CHD, most of whom have had a previous AMI. This difference (a functional measure of platelet reactivity) appears to be predicted by different factors in the two groups. Whether this is due to platelet factors and/or environmental effects remains uncertain.

There was no measurable difference in the release of BTG from alpha-granules following collection and processing of plasma samples in young males with or without CHD. Therefore, it is unlikely that an *in vivo* process is occurring that causes an ongoing release reaction within the circulation without provocation in young males with stable CHD at a time when there is no evidence of acute thrombotic occlusive manifestations of CHD. It is unlikely that the classic CHD risk factors exacerbate or cause platelet hyper-reactivity involving alpha-granule proteins, although plasma fatty acids may do so. The results demonstrated the presence of a qualitative difference in the relationship between platelet function and plasma fatty acids between young males with CHD and a normal control group.

Thromboxane production in clotted whole blood in the case group (with CHD) appears to be quantitatively and qualitatively different from the control group. There were different influences on TXB₂ production due to various plasma constituents, suggesting that an alteration in the reactivity of the platelet eicosanoid system exists

in young males with CHD. Blood cell indices appear to play an important role, as well as the plasma fibrinogen level and the degree of previous tobacco intake.

In the case group, the most reliable predictor of serum TXB2 levels was a platelet parameter, (viz MPV), rather than external factors in whole blood. In contrast, in the control group, serum TXB2 was most reliably predicted by factors within the blood, external to the platelet. That is, external influences within the milieu of the clot, and in particular, specific plasma fatty acids and the blood glucose level influence TXB2 production in the control group.

The young males with premature CHD had different *in vitro* aggregation responses from normal controls, in response to adrenaline. Again, as for other platelet function measures, the aggregation responses were influenced by different factors in the two groups. Aggregation in the control group was positively influenced by the platelet number in the plasma and negatively by the red cell volume in the blood. In the case group, aggregation was directly influenced by thromboxane metabolism, by the WCC and by apoprotein A1 levels.

These results support the concept that the platelets of males with premature CHD function differently to those without clinical atherosclerosis, and that this may contribute adversely to *in vivo* thrombus formation. The results provide further support for recent findings that platelet aggregation is predictive of cardiac events and CHD mortality. This concept is further strengthened by our observation that there is a significant relationship between the severity of CAD and adrenaline-induced platelet aggregation in young males with premature CHD.

9.5.8 Fibrinogen, CHD and Angiographic CAD

Elevated fibrinogen levels do have a modest relationship with severe CAD. This relationship, however, may be due to another process simultaneously leading to increased fibrinogen levels and the development of severe stenotic CAD. The results suggested that any relationship existing between fibrinogen and CAD is unlikely to be due to a non-specific response to atheromatous involvement of the coronary arteries. An independent association between fibrinogen and the extent of coronary arterial wall involvement, and by inference, atherogenesis, was not able to be demonstrated.

The fibrinogen level for an individual with CAD is in part directly predicted by the volume of previous cigarette use, the age of the individual, the severity of the underlying CAD, and inversely by the HDL cholesterol level. Fibrinogen levels are higher in young male patients after the diagnosis of CHD than in controls without CHD. The elevated fibrinogen is not associated with angiographically defined CAD in the young CHD group. Factors associated with the fibrinogen levels differ between controls and cases, with a cluster of factors, viz blood glucose level, HbA1C, BMI, body weight and scapular skinfold thickness, being associated with fibrinogen levels in the case group. An inverse association between HDL cholesterol and fibrinogen levels in patients with CHD was demonstrated in preparatory study of this Thesis and confirmed in the main case-control study. This was a novel observation which has also been reported independently by others. The mechanism of this association

remains to be elucidated. Moreover, in male CHD patients with premature presentation, the fibrinogen level appears to have an association with other more recently recognised risk factors which cluster together, viz central body obesity, abnormal glucose homeostasis and lipid abnormalities.

The results remain consistent with the observation that *a priori* high fibrinogen levels may be predictive of an increased incidence of CHD events. This predictive relationship would appear to be mediated through an association with other risk factors and with CAD severity. Whether there is any causal relationship between CHD events and fibrinogen levels still remains to be determined.

9.5.9 Behavioural and Psychological Measures in Males with CHD

In the two groups of CHD patients evaluated in this study, a reproducible univariate correlation between the MMPI hostility score and CAD has been demonstrated. The relationship, however, was not sufficiently strong, in comparison to other risk factors or because of an association with the CHD risk factors, to remain independently predictive of CAD following multivariate analysis (Appendix 3).

9.5.10 Plasma Fatty Acid, Platelet Function and CHD

The observations in this Thesis indicate that for males with premature CHD, *in vitro* platelet aggregability is in part influenced by the plasma fatty acid composition. Distinct differences in plasma fatty acid and the relationship to platelet aggregation have been demonstrated for the two relatively homogenous groups studied. The CHD group have different platelet aggregation responses in comparison to the control group (Appendix 1).

9.5.11 The Relationship Between Body Fat Distribution, Plasma Fatty Acids and Angiographically Defined CAD

The absence of an association between HbA1C and blood glucose levels and diastolic BP with central body fat in the control group, and the presence of this association in the case group in the present study, supports the possibility of an aetiological relationship with altered glucose homeostasis, hypertension, central obesity, plasma fatty acid and abnormal lipoproteins, and CHD. The distribution of subcutaneous fat, as measured by triceps and scapular skinfold thickness, appears to differ between young males with and without CHD who have the same overall body mass measurement. In addition, young non-diabetic males with CHD have increased measures of glycosylation of plasma proteins, viz HbA1C. An association between a measure of central obesity and measures of blood glucose and HDL cholesterol level are observed only in the group with CHD. In the same group, the glycosylation appears to be predicted more by the plasma content of C18 group of fatty acids. The extent of angiographically defined CAD is better predicted by the scapular skinfold thickness and myristic acid than other risk factors for CHD which are associated with the measure of central body obesity (Appendix 2).

The combination of abnormal glucose homeostasis, truncal obesity and lipoprotein abnormalities imparts significant risk for developing CHD. We have demonstrated

that plasma fatty acid content may contribute to this relationship.

9.5.12 Angiographically Defined CAD and Risk Factors for CHD

The results of this study indicate that the formation of acute severe stenoses has different risk factors and mechanisms than those involved in the development of diffuse atherosclerosis, as has been previously proposed.^{4,623} The observations support the concept of different factors influencing different stages in the development of the clinical condition. In young males with CHD, specific factors are associated with the atherosclerotic component and other factors with the stenotic component, and some with both. In young males with CHD, platelet function plays an important part in determining the severity of the CAD. Fatty acids, lipoproteins and body fat distribution play a major part in determining intramural involvement of coronary arteries by atherosclerosis, as defined angiographically. In particular, no platelet function measure was related to the extent of disease.

9.5.13 Conclusion

The results of this thesis contribute to the criteria required to establish causality of a factor in epidemiological terms. Firstly, potentially heightened platelet activation is present more often and in greater degree in the group of prevalent CHD cases than in a control group without CHD. Secondly, there is a biological gradient in the predicted disease outcome, namely severity and number of discrete obstructive lesions and the aggregability of platelets to adrenaline. The overall body of literature currently provides a strong argument that a latent potential for increased platelet aggregation may be a causative risk factor for the clinical expression of CAD as manifested in the thrombotic complications.

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APPENDICES

APPENDIX 1

PLASMA FATTY ACIDS AND PLATELET REACTIVITY IN MALES WITH PREMATURE CORONARY HEART DISEASE

A1.1 PLASMA FATTY ACID PROFILE AND PLATELET FUNCTION

A1.1.1 Introduction

That dietary fats probably play an important role in the pathogenesis of CHD is generally accepted.^{67,150,162,408,1019-1021} Studies have concentrated on the relationship between diets rich in saturated fats, blood cholesterol and CHD. Many years after the initial observations,¹⁰²²⁻¹⁰²⁴ the possibility that a deficiency of essential fatty acids (FA) may play a role in the development of atherosclerosis and CHD has reemerged.^{379-381,1025,1026} In support of this, two prospective epidemiological studies have suggested that a diet high in polyunsaturated fats may reduce the risk of CHD.^{1020,1027}

Those reports indicating a relationship between body tissue fatty acids composition and CHD are persuasive but not conclusive. It is clear that more information is needed to support the concept and provide a biological explanation for the relationship. The consistent association between low tissue levels of polyunsaturated fatty acids (PUFA) and CHD, in both individuals and populations, does implicate an association with CHD and possibly environmental and/or metabolic factor(s) related to body tissue FA composition. Since tissue FA reflect the long-term intake of polyunsaturated fats in the diet,¹⁰²⁶ dietary variations of PUFA may be one environmental factor. Abnormal FA metabolism (e.g. peroxidation) may be another factor.¹⁰²⁶ Alternatively, changes in haemostatic factors may be important since they offer a plausible biological link between FA metabolism and CHD.^{35,36,1023} In the light of current knowledge of the thrombotic aetiology of AMI,³ the association may be through the occurrence of arterial thrombosis. The relationship between FA, platelet reactivity and arterial thrombosis has been extensively studied.^{66,67,624,625,657,659,862,971,1028-1036}

Even though a large number of important studies have implicated reduced levels of certain PUFA in body tissues as being risk indicators for the development of CHD,^{379-381,818,1022,1024-1026,1037-1039} public health measures aimed at altering the proportion of PUFA in the diet in order to modify blood lipid levels of a population may not necessarily have a favourable effect on platelet function.^{645,646,1036} Indeed, there may be critical upper and lower limits for the content of dietary n-6 PUFA in order to induce beneficial effects on platelet function and thrombosis.^{645,646,1036}

It is clear that the effects of the various PUFA on thrombosis and atherosclerosis, and in particular the n-6 PUFA, have only partly been elucidated.⁶⁷ If dietary modification of the tissue FA is to be advised as a primary and secondary preventative measure against CHD, a more complete understanding of the effects of fatty acids on platelets and coagulation is required in addition to effects on blood lipids. In this section, the relationship between the total FA content of plasma and *in vitro* platelet aggregation in the group of young males with CHD and the age, sex and socioeconomically matched control group, are examined. This evaluation is of importance given the difference in platelet function demonstrated between the case and control groups.

A1.1.2 Methods

A1.1.2.1 Study populations. The patient population consisted of the case-control study groups described in Chapter 3.

A1.1.2.2 Lipoprotein measurement. The methods are described in Chapter 5.

A1.1.2.3 Platelet aggregation. The materials and methods used for platelet aggregation measures are described in Chapters 2 and 6.

A1.1.2.4 Plasma fatty acid analysis. Plasma samples were stored at -70°C prior to analysis. Lipids were extracted from 1ml aliquots of plasma with 9 ml of chloroform-methanol (2:1, v/v) and 2.5ml KCl. The lipid extract was filtered to remove protein and then evaporated to dryness with nitrogen gas. Lipids were then hydrolysed and methylated with 1ml of 14% boron trifluoride in dry methanol at 100°C for 2 min. Once the solution reached ambient temperature, 5 ml of water was added followed by 5 ml of hexane to extract the methyl esters which were then brought up to 40 ul of methylene chloride/butylated hydroxy toluene solution. The FA methyl esters were separated by gas-liquid chromatography (GLC) using a 30m x 0.53mm I.D. cyanopropyl megabore column and a flame ionisation detector. Nitrogen was the carrier gas, column conditions were optimised to separate the peaks of interest and the temperature was programmed at 210°C to run isothermally. Peaks were identified by comparison with chromatography of authentic FA methyl ester mixtures. The standards of FA methyl esters were obtained from Supelco, Inc. (Bellefonte, PA 16823-0048) and Alltech Associates (Deerfield, IL 60015). A Hewlett Packard model 3396A integrator was used to calculate the % composition of each FA methyl ester peak.^{1040,1041}

To assess the reliability of the methylation process the area under the GLC peak of a methylated standard of known concentration was compared to that peak from the same FA with known concentration and which was subsequently methylated. A standard mixture of 50mg FA methyl ester (Alltech Associates, cat. no. 19026-26) was brought up in 1ml of methyl chloride in BHT. In the standard there were 5 components (C13:0, C15:0, C17:0, C19:0 and C21:0) each of 10mg/ml concentration. Then 10mg of heptadecanoic acid was methylated at 100°C for 2 min with 1ml of BF₃/MeOH. The methyl ester was brought up in 1ml of methylene chloride and BHT. Both 1ul of the standard and the sample were injected separately (at 170 to 200°C, R°C per min, Inj=250°C, det=260°C and attenuation=3). The resulting chromatograms were analysed to calculate the % methylation of FA:

$$\begin{aligned}\% \text{ methylation} &= \frac{\text{area of newly methylated C17:0 peak}}{\text{area of pre-methylated standard C17:0}} \times 100 \\ &= \frac{39.57}{40.12} \times 100 \\ &= 98.63\%\end{aligned}$$

To assess the precision of the measurements, replicate measurements with standard FA were undertaken with separate samples from the same mixture of 4 different

standard FA (C20:0, C20:1, C20:2, and C20:3). The samples were run through the GLC 10 times each. The CV ranged from 0.36% to 0.53% (Table A1.1).

The present literature quotes coefficients of variation (CV) for major component fatty acids between 0.9% and 4.4%, with greater variation for trace components (content<1%), of the order of 17% to 35%^{379,1026} Variability in the results were determined for the error of the method (replicates), and variability for repeated identical sample measures, which includes method variability over time, and also within-person variation over time. Replicates of the same blood sample demonstrated a CV between 0.9% and 6.2% for the major components, and above 7% for the trace components (content<1%) (Table A1.2). The CV for C20:1w9 and C20:2w6 were unacceptably high but commensurate with the literature,^{379,1026} whilst the remaining FA had CV<10% (Table A1.2). Therefore, caution will need to be exercised in the interpretation of results involving C20:1w9 and C20:2w6 with the methods used.

A1.1.2.5 Statistical analysis. The statistical methods are described in Chapter 2.

A1.1.3 Results

A1.1.3.1 Group comparisons. Compared to the control group, the CHD group had a higher % of palmitic acid in plasma, and a lower % of oleic, eicosenoic and docosapentanoic acids and a lower % of linoleic acid which was of borderline significance (Table A1.3).

A1.1.3.2 Correlations between plasma fatty acids and platelet aggregation. When associations were evaluated by linear correlation, an increased % of palmitic, myristic, alpha-linolenic, docosahexanoic, oleic and eicosenoic acids in plasma were found to be associated with a reduction in *in vitro* platelet aggregation in the CHD group (Table A1.4). In contrast, the % of stearic, dihomo- γ -linolenic and arachidonic acids correlated positively with some measures of platelet aggregation (Table A1.5). In the control group, only the % of myristic (LAGCOL $r=-0.31$, LT50ADR $r=-0.30$, LT50COL $r=-0.30$, RADR $r=0.28$, all with $p<0.05$), palmitic (RADR $r=0.33$, $p<0.05$), and dihomo- γ -linolenic acids (LT50ADR $r=-0.32$, RADR $r=0.35$, both with $P<0.05$) in plasma were associated with increased platelet aggregation. The % of stearic (LT50ADR $r=0.40$, LT50COL $r=0.27$, RCOL $r=-0.28$, all with $p<0.05$) and eicosenoic acids (LAGCOL $r=0.49$, LT50COL $r=0.50$, RCOL $r=-0.36$, all with $p<0.05$) in plasma were associated with decreased platelet aggregation in the control group.

A1.1.3.3 Multivariate analysis for aggregation measures and plasma FA. In both study groups all of the FA which linearly correlated with platelet aggregation, or differed between the two groups, were entered into a stepwise regression model. In the case group, stearic, dihomo- γ -linolenic and arachidonic acids retained a positive and independent association with measures of platelet aggregation, while docosahexaenoic and oleic acids remained independently associated with decreased aggregability (Table A1.5). In contrast, in the control group, myristic acid alone remained positively associated with increased platelet aggregability (LT50COL $B=-0.001$, Beta=-0.37; LAG $B=-0.001$, Beta=-0.38; RCOL $B=0.001$, Beta=0.46; all

with $P < 0.006$; and RADR $B = 0.002$, $Beta = 0.35$, $p < 0.03$) and eicosenoic acid was associated with decreased aggregation (LT50COL $B = 0.001$, $Beta = 0.45$; LAG $B = 0.001$, $Beta = -0.47$; RCOL $B = -0.001$, $Beta = -0.32$; all with $p < 0.02$). The platelet count in PRP (PRPPC) remained positively associated with the rate of aggregation in the control group (RCOL $B = 0.001$, $Beta = 0.32$; RADR $B = 0.001$, $Beta = 0.35$; both $p < 0.01$).

A1.1.3.4 Multivariate analysis of platelet aggregation and blood lipids. In light of the possible interaction between FA in various lipids and in platelets, each of the main lipoprotein indices (cholesterol, triglycerides, HDL, apoprotein B and apoprotein A1) and PRPPC were analysed by multiple regression as independent variables to determine any potential relationship with aggregation, with the measures of aggregation being the dependent variable. Only cholesterol and apoprotein A1 retained a possible independent relationship with some of the measures of aggregation. These variables were entered into the final regression model. There was no effect on outcome of predicting independent variables with the estimated amount of alcohol in grams being entered in the regression analysis as an independent variable in the control group. Therefore it was excluded from subsequent analysis to reduce the number of factors assessed.

A1.1.3.5 Multivariate analysis of platelet aggregation, fibrinogen, plasma FA and blood lipids. Multiple regression analysis was performed with those same FA linearly associated with aggregation or differing between the groups, in addition to total cholesterol, apoprotein A1 and fibrinogen as potentially confounding factors. Stearic and dihomo- γ -linolenic acids remained positively associated with platelet aggregability, while palmitic acid became positively associated with platelet aggregation. Oleic acid remained associated with decreased platelet aggregation in the CHD group (Table A1.6). In the control group, PRPPC and myristic acid remained associated, and arachidonic acid became associated with increased platelet aggregation using the same regression model (Table A1.7). Both stearic and eicosenoic acids retained a relationship with decreased platelet aggregation. Thus, a qualitative difference in the relationship between stearic acid and platelet aggregation exists in the two study groups. Males with premature CHD have an increased aggregation response in association with an increased % of stearic acid, whereas there was the opposite relationship in the young male control group.

A1.1.3.6 Classes of plasma FA - group comparisons. The % content of the FA were categorised into the classes n-3, n-6, n-9, saturated FA (SFA), and oleic plus docosahexanoic acids (OL+DH). The eicosapentanoic to arachidonic acid content ratio (EPA/ARA) and PUFA/SFA ratios were also calculated. Predictably there were significant differences between the two groups (Table A1.8). The values for n-9, P/S and OL+DH were significantly higher in the control group compared to the case group, while the value for n-6 was of borderline significance. The SFA were significantly higher in the case group (Table A1.8).

A1.1.3.7 Classes of plasma FA - correlations. In the case group, the values for n-9 and OL+DH had consistent negative associations with platelet aggregation, and for n-6 a positive relation with increased aggregation (Table A1.9). No consistent associations were demonstrated in the control group.

A1.1.3.8 Classes of plasma FA - multivariate analysis. When the values for n-3, n-6, n-9 and SFA classes of FA were analysed with multiple regression analysis, the n-6 and n-9 classes were found to have an independent association with some measures of platelet aggregation, in the case group. There was a positive association with increased aggregation in the n-6 class, while in the n-9 class there was a positive association with decreased aggregation (Table A1.10). The relationships appeared to be related to the rates of aggregation (Table A1.10). In the control group, none of the FA classes or ratios were independently associated with aggregation when assessed by multiple regression analysis. Aggregation in the control group was independently and positively associated with the PRPPC for RADR ($B=0.002$, $Beta=0.71$, $p<0.005$) and RADP ($B=0.001$, $Beta=0.42$, $p<0.05$).

A1.1.4 Discussion

Distinct differences have been shown in the qualitative relationships between plasma FA and *in vitro* platelet aggregation in response to specific agonists in males with premature CHD. In fact, cases and controls had different plasma FA profiles and specific FA were associated with differences in platelet aggregability independent of established risk factors. The % content of stearic, arachidonic and dihomo- γ -linolenic acid were associated with increased platelet aggregability. Additionally, palmitic, oleic and docosahexanoic acids were associated with decreased platelet aggregability. Except for arachidonic and docosohexanoic acids, these same FA continued to be independently associated with platelet aggregation with the inclusion of other appropriate risk factors for CHD and lipids into the multivariate regression model for CHD patients. Palmitic acid, however, became positively associated with increased aggregation. In contrast, using the same analysis, myristic and arachidonic acids were associated with increasing, and stearic and eicosenoic acids with decreasing platelet aggregation in the control group. This latter observation is consistent with current epidemiological and experimental evidence regarding the effects of saturated FA on platelet function,^{67,1029,1042} but notably different to the CHD group. Of particular consequence, the n-6 FA as a group were associated with increasing, and n-9 with decreasing platelet aggregation in the CHD group, but not in the controls.

A1.1.4.1 Saturated fatty acids. Both experimental^{624,862,1032-1034} and epidemiological observations^{659,1030,1031,1036} have demonstrated the thrombogenic potential of saturated FA, which may in part be mediated through increased platelet reactivity.^{67,625,971,1029} In the control group, the observed association between increasing plasma myristic acid and the aggregability of platelets is consistent with the published data.^{67,659,769,1030-4,1036} However, in the patient group, stearic and palmitic acids were associated with increased platelet aggregability after adjustment for other variables.

An explanation for the differences may relate to the fact that the % distribution of FA in body tissues and plasma lipids is influenced by many external factors.^{379,863,900,1043,1044} In particular, the dietary FA content has a considerable influence on the body FA content including the distribution of FA in plasma.^{645,659,1025,1031,1045-1047} Many of the FA measured are metabolic products of shorter chain FA, and in humans the production of longer chain PUFA requires the dietary intake of essential precursors. Therefore, any significant linear correlations

between FA and platelet aggregation may be confounded by the relationships between the FA themselves. For example, an increase in stearic acid may be at the expense of other saturated fats such as myristic and palmitic acids. In such circumstances, if an increase in stearic acid is associated with increased platelet aggregation, then myristic and palmitic acids may inappropriately appear to correlate with decreased platelet aggregation. Multiple regression analysis was used to adjust for such confounding influences. In fact, with multiple regression analysis, myristic acid was found not to be independently associated with decreased platelet aggregation in the CHD group, while palmitic acid became positively associated with aggregation, and stearic acid maintained a positive independent association.

An association between stearic acid and thrombogenicity has been repeatedly demonstrated,¹⁰³²⁻¹⁰³⁵ but its association with platelet aggregation is contradictory.^{624,862,1028} Our results indicate the presence of a qualitative difference in the relationship between stearic acid and platelet aggregation in the two relatively homogeneous study groups. Previous inconsistencies in the literature may have arisen in part from the use of heterogeneous groups. The relatively homogeneous group of CHD patients evaluated in this study has a different platelet response to agonists compared to controls, and the measures of aggregation differ in their association with total plasma FA content in the two groups. In particular, males with premature CHD have an increased aggregation response in association with an increased % of stearic acid, whereas there was a contrary relationship in the young male control group.

A1.1.4.2 Unsaturated fatty acids. Linoleic acid appears to be less thrombogenic than saturated fats in animals and humans.^{66,67,862,1035} Reduced platelet aggregability has been reported in response to high linoleate diets^{67,660,971} which effect the fatty acid profile of body tissues.⁶⁷ However, other studies have raised the prospect of a positive association existing between a high intake of vegetable fat and increased platelet aggregability.^{645,646,1036} Linoleic acid in both adipose tissue and plasma triglycerides has been reported to correlate positively with ADP-induced platelet aggregation.¹⁰³⁶ This supports the possibility of an increase in platelet reactivity to ADP and adrenaline following an increase in dietary linoleic acid.⁶⁴⁵ In addition, increases in BTG and ADP-induced platelet aggregation have been observed in association with an increase in the PUFA ratio.⁶⁴⁶ Both high and low levels of linoleic acid may have adverse effects on thrombosis.^{67,645,646,861,1036} In our study, no association was demonstrated between platelet aggregation and plasma linoleic acid content in either the control or the patient group. However, a linear relationship was demonstrated between the plasma content of metabolic products of linoleic acid, namely dihomo- γ -linolenic and arachidonic acids in the CHD group and dihomo- γ -linolenic acid in the control group, and increasing platelet aggregability. Indeed, with multiple regression, the plasma content of dihomo- γ -linolenic acid maintained an independent association in the case group, and arachidonic acid content in plasma was associated in the control group with increasing platelet aggregation.

Dihomo- γ -linolenic, linoleic acid and eicosapentanoic acid enriched diets in humans have generally been associated with inhibition of platelet aggregation and decreased TXB₂ formation.⁶⁶ Nevertheless, feeding purified dihomo- γ -linolenic acid to humans or animals has resulted in conflicting results with respect to effects on platelet function.⁶⁶ Naturally occurring fats and oils contain only minute amounts of dihomo- γ -

linolenic acid and to increase the concentration in plasma or tissues, artificially enriched fats need to be administered.⁶⁶ However, population based studies have shown significantly lower levels of dihomoy-linolenic acid in newly diagnosed CHD patients¹⁰²⁶ and populations at increased risk of developing CHD.³⁸⁰ Furthermore, it is feasible that increasing levels of linoleic acid intake may result in greater tissue levels of dihomoy-linolenic and arachidonic acids leading to adverse effects on platelet aggregability. In addition, from an understanding of the biochemical actions of arachidonic acid, an increased plasma content of arachidonic acid could be associated with increased platelet aggregation. Indeed, a reduction in cellular phospholipid content of arachidonic acid has been shown to result in an inhibition in platelet aggregation.⁶⁶

An important observation in the present study was that after the FA were grouped together, the n-6 FA as a group maintained an independent association with increased platelet aggregability in the case group. Our observations underscore contemporary statements against indiscriminate dietary supplementation with linoleic acid,⁶⁷ notably in CHD patients.

A1.1.4.3 Monounsaturated fatty acids. Previous studies have shown a positive relationship between plasma content of oleic acid, and increased platelet aggregation,^{862,1030} and increased clotting.¹⁰³⁵ In another study a positive association was noted between the plasma concentration of saturated FA, oleic acid and platelet aggregation.¹⁰³⁰ Despite these initial observations, other studies have failed to demonstrate a similar results.^{67,1029,1036} The magnitude of platelet aggregation induced by collagen in healthy individuals fed a low fat diet rich in oleic acid is lower when compared to those fed a similar diet rich in n-6 PUFA.¹⁰⁴⁶ Under experimental conditions, oleic acid does not appear to be thrombogenic, and, indeed may be anti-thrombogenic.¹⁰³⁵ Decreased levels of oleic acid in plasma were observed in the present study in the CHD group, and the plasma content was negatively associated with measures of platelet aggregability. In the control group oleic acid had no major influence on *in vitro* platelet aggregation. The negative finding in normal individuals is consistent with most,^{67,1029,1035,1036,1046} but not all studies.^{862,1030}

One possible explanation for the difference described may relate to the beneficial effect of oleic acid on oxidation of LDL since oxidised LDL increases platelet aggregation.¹³⁴ A diet enriched in oleic acid generates oleic acid rich LDL particles which are resistant to oxidation.¹⁰⁴⁸ Whatever may be the explanation for the different association between oleic acid and platelet aggregation observed in this study, the qualitative difference in platelet aggregation between the two groups is further highlighted.

A1.1.4.4 n-3 polyunsaturated fatty acids. The n-3 PUFA have consistently been found to have anti-thrombogenic and anti-platelet effects, in particular eicosapentanoic and docosahexaenoic acids.^{66,67} Ingestion of fish oil rich in n-3 PUFA has profound effects on platelet and vessel wall composition and function.^{67,1049} Associated with a decrease in cellular phospholipid concentration of arachidonic acid, the bleeding time is prolonged and *in vitro* platelet function is inhibited.⁶⁶ The platelet lipid content of eicosapentanoic acid and the EPA/ARA ratio correlate inversely with ADP-induced aggregation.¹⁰³⁶ This observation is consistent with the decreased

reactivity of platelets associated with a high EPA/ARA ratio in Eskimos,¹⁰⁴⁹ and in individuals fed a mackerel diet.¹⁰⁵⁰ Indeed, the EPA/ARA ratio of plasma FFA may be a risk factor for CHD.¹⁰³⁹ In addition, the plasma cholesterol and triglyceride content of eicosapentanoic acid and the EPA/ARA ratio have an inverse correlation with adrenaline-induced platelet aggregation.¹⁰³⁶

The relative amounts of the n-3 PUFA in plasma is small in both of our study groups. In the control group the n-3 PUFA did not have any relationships with platelet aggregability. This is not an unexpected observation since the platelet inhibiting effects of n-3 PUFA is dose related and in general increased levels appear to be needed for the effect to be produced in clinical studies.^{66,67,1036,1049,1050} In the patient group the apparent inverse relation between % docosahexanoic acid in plasma and platelet aggregation is an association which appears to not be independent of other factors.

A1.1.4.5 Fatty acid groups. On account of the number of variables examined, and because classes of FA may influence platelet aggregation more than individual FA, the FA were grouped into the classes, viz n-3, n-6, n-9 and SFA. In addition, the P/S and EPA/ARA ratios were also evaluated. The strongest independent relationship observed for the grouped FA was with the n-6 class which was positively associated with increased platelet aggregation, and the n-9 class which was associated with decreased aggregation, a finding anticipated from the observations with the individual FA.

A1.1.5 Summary

1. The observations in this section indicate that for males with premature CHD, *in vitro* platelet aggregability is in part influenced by the plasma FA composition. Many of the published studies are similar to several of our observations in a young male control group, making it probable that the current observations are not spurious. The variability in results noted to date in many studies^{66,67} may arise from various causes, including methodological differences and variations due to heterogeneous populations.

2. Distinct differences in plasma FA and their relationship with platelet aggregation have been demonstrated in the two relatively homogeneous groups studied.

3. The possibility that alternate avenues of fatty acid metabolism result in the differences in the functional platelet measures arises from the observations in the above evaluation. Variation in fatty acid metabolism may therefore contribute to the difference in platelet function between the two groups and be a primary thrombogenic risk factor for CHD. This potentially confounding influence on the results of the case-control study indicate the importance of including plasma fatty acid content in the final analysis.

Table A1.1. Replicate measurements of fatty acids (n=10).

	C20:0	C20:1	C20:2	C20:3
Mean	25.98	26.95	24.93	22.14
Maximum	26.14	27.11	25.09	22.31
Minimum	25.69	26.78	24.80	21.99
SD	0.13	0.10	0.11	0.12
95% conf	0.08	0.06	0.07	0.07
99% conf	0.11	0.08	0.09	0.10
Variance	0.02	0.01	0.01	0.01
CV (%)	0.51	0.36	0.42	0.53

Ten replicate measures were made of known standards to assess the precision of the GLC methods. The mean of the samples, the maximum and minimum values, the standard deviation (SD), the 95% and 99% confidence intervals (conf), the variance and the coefficient of variation (CV) are provided.

Table A1.2. Fatty acid replicates measured from the same blood sample (n=14).

	C14:0	C16:0	C16:1w7	C18:0	C18:1W9	C18:2w6	C18:3w6
Mean	2.12	29.59	3.58	12.27	20.19	20.48	0.44
Max	2.25	30.34	3.80	12.53	20.57	20.87	0.50
Min	1.96	28.51	3.23	12.04	19.88	20.20	0.37
SD	0.09	0.52	0.22	0.15	0.19	0.19	0.04
95% CI	0.05	0.27	0.12	0.08	0.10	0.10	0.02
99% CI	0.07	0.36	0.15	0.11	0.13	0.13	0.03
Variance	0.01	0.27	0.05	0.02	0.04	0.03	0.00
CV (%)	4.43	1.76	6.15	1.26	0.95	0.90	9.30

	C20:1w9	C20:2w6	C20:3w6	C20:4w6	C20:5w3	C22:5w3	C22:6w3
Mean	0.19	0.29	2.25	5.67	0.57	0.62	1.25
Max	0.29	0.42	2.51	5.94	0.61	0.78	1.42
Min	0.10	0.20	2.20	5.39	0.44	0.53	1.15
SD	0.06	0.07	0.09	0.04	0.01	0.02	0.02
95%CI	0.03	0.04	0.05	0.08	0.02	0.04	0.04
99%CI	0.04	0.05	0.06	0.11	0.03	0.05	0.06
Var	0.00	0.00	0.00	0.03	0.00	0.00	0.01
CV(%)	32.98	24.12	3.83	2.77	7.68	10.77	6.41

See Table A1.1 for abbreviations and Table A1.5 for FA names corresponding to the FA abbreviations. Var=variance, CI=confidence interval, SD=standard deviation.

Table A1.3. Plasma fatty acid composition in both groups.

Fatty Acid		Control Group	Case Group
Myristic	(C14:0)	2.61+/-1.32	2.85+/-1.36
Palmitic *	(C16:0)	23.51+/-4.59	26.16+/-3.79
Palmitoleic	(C16:1n7)	2.31+/-1.00	2.25+/-0.76
Stearic	(C18:0)	12.91+/-2.21	12.97+/-1.54
Oleic **	(C18:1n9)	22.02+/-3.35	19.91+/-2.91
Linoleic #	(C18:2n6)	20.76+/-3.60	19.59+/-2.96
alpha-Linolenic	(C18:3n6)	0.57+/-0.33	0.58+/-0.34
Eicosenoic ##	(C20:1n9)	0.99+/-0.83	0.51+/-0.24
Eicosadienoic	(C20:2n6)	0.50+/-0.29	0.42+/-0.18
Dihomo-γ-linolenic	(C20:3n6)	2.21+/-0.76	2.27+/-0.74
Arachidonic	(C20:4n6)	7.45+/-2.15	7.22+/-1.71
Eicosapentaenoic	(C20:5n3)	0.84+/-0.53	0.86+/-0.42
Docosapentaenoic ~	(C22:5n3)	1.05+/-0.39	0.90+/-0.36
Docosahexaenoic	(C22:6n3)	2.77+/-1.11	2.98+/-1.01

*p=0.0028, **p=0.0015, #p=0.0861, ##p=0.0002, ~p=0.0543

Mean % plasma content expressed as mean +/- SD.

Table A1.4. Linear correlations between fatty acids in plasma and platelet aggregation measures in the case group.

	C14:0	C16:0	C18:0	C20:3n6	C20:4n6	C22:6n3	C18:1n9
LAGCOL	0.21	0.27#	-0.26#	-0.19	-0.13	0.11	-0.03
LT50ADR	0.17	-0.02	-0.44~	-0.43~	-0.13	0.37*	0.02
LT50ADP	0.14	0.02	-0.38~	-0.21	-0.54~	0.04	0.13
LT50COL	0.33*	0.31*	-0.24#	-0.13	-0.22	0.15	-0.01
RADR	-0.22	-0.13	0.33*	0.35*	0.18	-0.43~	-0.20
RADP	-0.09	-0.10	0.53~	0.28#	0.41~	-0.25#	-0.31*
RCOL	-0.02	-0.12	0.30*	0.04	0.28#	0.01	-0.25#

	C18:2n6	C18:3n6	C16:1n7	C20:1n9	C20:2n6	C20:5n3	C22:5n6
LAGCOL	0.03	-0.04	0.09	-0.06	0.02	-0.17	-0.09
LT50ADR	-0.05	0.33#	0.17	0.34*	-0.03	-0.07	-0.16
LT50ADP	0.24	0.03	0.04	-0.01	-0.12	-0.09	-0.15
LT50COL	0.10	0.01	0.04	-0.05	0.06	-0.06	0.07
RADR	0.19	-0.18	-0.16	-0.05	0.08	0.27	0.18
RADP	0.19	-0.37*	-0.19	-0.09	0.07	0.19	-0.04
RCOL	-0.13	0.19	-0.01	-0.09	0.02	0.05	-0.05

p<0.05, * p<0.02, ~ p<0.005

See section A1.1.2.3 for the abbreviations for the aggregation measures and Table A1.1 for the names of FA.

Table A1.5. Multiple regression with FA as independent variables and aggregation measures as dependent variables in the case group.

Dependent Variable	Independent Variable	B	SE B	Beta	Rsqr	Sig.
LT50COL	C18:0	-0.0005	0.0002	-0.4915	0.2004	0.0036
	C22:6n3	0.0006	0.0003	0.3422	0.3151	0.0356
LAG	C18:0	-0.0005	0.0002	-0.4055	0.1645	0.0213
RCOL	C18:0	0.0013	0.0003	0.8187	0.2660	0.0002
	C20:3n6	0.0016	0.0007	0.4545	0.3808	0.0277
LT50ADR	C20:3n6	-0.0016	0.0005	-0.5849	0.2546	0.0013
	C22:6n3	0.0008	0.0004	0.3677	0.3834	0.0311
RADR	C22:6n3	-0.0014	0.0004	-0.5446	0.2420	0.0012
	C18:0	0.0008	0.0002	0.4560	0.4563	0.0043
LT50ADP	C20:4n6	-0.0003	0.0002	-0.4741	0.4561	0.0241
RADP	C18:0	0.0008	0.0003	0.4251	0.3319	0.0122
	C18:1n9	-0.0004	0.0002	0.3313	0.4189	0.0460

Only FA having a linear relationship with aggregation were entered (viz C14, C16, C18, C18:1n9, C18:2n6, C20:1n9, C20:3n6, C22:5n3 and C22:6n3).

Table A1.6. Multiple regression entering all associated FA, PRPPC, total cholesterol, apoprotein A1 and fibrinogen as independent variables in the case group, with aggregation measures as the dependent variables.

Dependent Variable	Independent Variable	B	SE B	Beta	Rsqr	Sig.
LT50COL	C18:0	-0.0005	0.0002	-0.5594	0.3130	0.0045
LAG	C18:0	-0.0007	0.0003	-0.4741	0.2248	0.0193
RCOL	Apo A1	0.0044	0.0012	0.5471	0.3168	0.0015
	C18:1n9	-0.0003	0.0002	-0.3374	0.5077	0.0386
	PRPPC	0.0011	0.0005	0.3329	0.6039	0.0395
LT50ADR	C20:3n6	-0.0020	0.0006	-0.7139	0.2268	0.0023
	Apo A1	0.0036	0.0013	0.3659	0.3555	0.0144
	C16:0	-0.0003	0.0001	-0.4779	0.5254	0.0405
RADR	Apo A1	0.0042	0.0014	0.5090	0.3741	0.0076
	C18:0	0.0006	0.0002	0.3945	0.5792	0.0316
RADP	C18:1n9	-0.0005	0.0002	-0.4700	0.4342	0.0012
	Apo A1	0.0041	0.0012	0.4260	0.6710	0.0030
	C18:0	0.0005	0.0003	0.2743	0.7311	0.0432

B=regression coefficient, Rsqr=coefficient of determination. Only FA having a linear relationship with aggregation were entered (viz C14, C16, C18, C18:1n9, C18:2n6, C20:1n9, C20:3n6, C22:5n3 and C22:6n3). See section A1.1.2.3 for the abbreviations for the aggregation measures and Table A1.1 for the names of FA. Only significant associations are shown. PRPPC=platelet count of PRP.

Table A1.7. Multiple regression entering all associated FA, PRPPC, total cholesterol, apoprotein A1 and fibrinogen as independent variables in the control group, with aggregation measures as the dependent variables.

Dependent Variable	Independent Variable	B	SE B	Beta	Rsq	Sig
LT50Col	C20:1n9	0.0012	0.0003	0.5508	0.3326	0.0009
	C18:0	0.0005	0.0002	0.4646	0.5478	0.0038
	C20:4n6	-0.0004	0.0002	0.4125	0.6612	0.0150
LAG	C20:1n9	0.0013	0.0004	0.6063	0.3677	0.0013
RCOL	C14:0	0.0010	0.0004	0.4994	0.2494	0.0110
RADR	PRPPC	0.0019	0.0005	0.6520	0.4923	0.0013
	C18:0	-0.0005	0.0002	-0.4276	0.6727	0.0190
RADP	PRPPC	0.0011	0.0005	0.4250	0.1806	0.0342

B=regression coefficient, Rsq=coefficient of determination. Only FA having a linear relationship with aggregation were entered (viz C14, C16, C18, C18:1n9, C18:2n6, C20:1n9, C20:3n6, C22:5n3 and C22:6n3). See Table A1.1 for names of FA corresponding to the abbreviations and section A1.1.2.3 for the aggregation measures. Only significant associations are shown. PRPPC=platelet count of PRP.

Table A1.8. Mean values of fatty acid classes and ratios in the two study groups.

	Controls (n=45)	Cases (n=49)	95% CI Diff Mean	P
n-3	4.66+/-1.64	4.74+/-1.41	-0.55 to 0.71	0.800
n-6	31.49+/-4.64	30.07+/-3.29	-3.06 to 0.22	0.088
n-9	23.09+/-3.45	20.41+/-2.90	-3.98 to -1.38	<0.001*
SFA	39.03+/-4.71	41.98+/-4.38	1.09 to 4.81	0.002*
EPA/ARA	0.12+/-0.08	0.12+/-0.06	-0.03 to 0.03	1.000
P/S	1.55+/-0.27	1.34+/-0.22	-0.31 to -0.11	<0.001*
OL+DH	24.79+/-3.10	22.89+/-3.01	-3.15 to -0.65	0.003*

* significant difference CI=confidence interval for the difference of the means. SFA=saturated fatty acids, EPA/ARA=eicosopentanoic acid/arachidonic acid, P/S=polyunsaturated fatty acids/saturated fatty acids, OL+DH=oleic plus docosohexanoic acids.

Table A1.9. Correlations the FA groups and ratios, and measures of platelet aggregation in the case group.

	n3	n6	n9	Sat	EPA/ARA	P/S	OL+DH
LAGCOL	0.01	-0.08	-0.03	0.20	-0.12	-0.19	0.02
LT50ADR	0.21	-0.20	0.23	-0.13	-0.01	0.08	0.31#
LT50ADP	-0.04	-0.10	0.13	-0.06	0.25	0.03	0.14
LT50COL	0.11	-0.04	-0.01	0.20	0.06	-0.21	0.05
REPI	-0.19	0.36*	-0.20	-0.06	0.23	0.08	-0.33#
RADP	-0.14	0.42~	-0.32*	0.08	-0.13	-0.01	-0.39~
RCOL	0.01	0.01	-0.25#	-0.01	-0.16	-0.06	-0.24#

p<0.05, * p<0.02, ~ p<0.005

Table A1.10. Multiple regression with values for n-3, n-6, n-9 and SFA, apoprotein A1, cholesterol, fibrinogen and PRPPC as independent variables and aggregation measures as dependent variables in the case group.

Dependent Variable	Independent Variable	B	SE B	Beta	Rsq	Sig
RCOL	Apo A1	0.0043	0.0012	0.5449	0.3168	0.0016
	PRPPC	0.0012	0.0005	0.3371	0.5068	0.0366
	n-9	-0.0003	0.0002	-0.3366	0.6038	0.0387
RADR	Apo A1	0.0050	0.0015	0.6117	0.3741	0.0032
RADP	n-9	-0.0007	0.0002	-0.5545	0.4526	0.0001
	n-6	0.00051	0.0001	0.5248	0.7140	0.0003

B=regression coefficient, Rsq=coefficient of determination. See Table A1.7 for the abbreviations of the FA groups and section A1.1.2.3 for the aggregation measures.

APPENDIX 2

BODY FAT DISTRIBUTION AND CORONARY HEART DISEASE

A2.1 THE RELATIONSHIP BETWEEN BODY FAT DISTRIBUTION, PLASMA FATTY ACIDS AND ANGIOGRAPHICALLY DEFINED CORONARY ARTERY DISEASE

A2.1.1 Introduction

Given the multifactorial aetiology of CHD and the degree of inter-dependence of the major risk factors, it is clear from the preceding discussions that the relative importance of risk factors as being independently associated with CHD, much less causative, can be difficult to discern. Obesity has been one such risk factor.¹⁰⁵¹ The association of obesity with CHD had been attributed to diabetes, hypercholesterolaemia and hypertension.^{371,387,390,642} More recent information, however, supports the hypothesis that obese individuals are independently at higher risk of developing CHD than persons of medium build.^{383,385,1052-1057} The nature of the association between obesity and cardiovascular disease is currently being extensively explored, and some clarification of the relationship is emerging,^{383,385,386,635,1055,1056,1058-1063} although much requires further investigation.¹⁰⁶⁴

The topographic distribution of fat appears to be more relevant as a risk factor for CHD than total body fat.^{383,384,386,1058-1065} An association between body fat distribution, risk factors for CHD and CHD itself has been recognised for many decades.¹⁰⁶⁶ Prospective studies have revealed a significant relationship between regional body fat distribution and cardiovascular disease incidence.^{1058,1059,1063} In addition, an increased proportion of trunk, or abdominal fat, appears to be associated with CHD mortality.¹⁰⁵⁸ Waist-to-hip ratio measurement^{1058,1067,1068} and scapular skinfold thickness^{1059,1063} are more closely related to CHD risk than measures of body weight or height.

The topographic distribution of adipose tissue is also associated with metabolic complications considered to be risk factors for CHD.^{383,1051,1059,1061,1066,1069} These include insulin resistance,^{1018,1069,1070} hyperinsulinaemia,^{1062,1069,1071} type II diabetes,^{1072,1073} hypertension,^{383,1069,1074-1077} altered blood lipoproteins,^{383,386,634,1051,1061,1071,1076-1079} and fatty acid metabolism.^{385,1080,1081}

A great deal of interest has been shown in the possible pathophysiology of these relationships.^{383,384,386,1061} Of particular interest is the evidence that upper-body fat is metabolically more active,^{383,395,1080,1081} and is more responsive to adrenergic agonists which stimulate lipolysis.³⁸⁵ This may lead to an excess release of free fatty acids^{1080,1081} which interfere with insulin clearance by the liver.^{383,385,1082} At present the significance of these associations in respect to the aetiology of CHD and of atherosclerosis remains uncertain,^{386,1051} although a number of interesting hypotheses have been proposed.^{383,385,635}

This study evaluates the relationship between measures of relative body weight and upper-body fatness, the major risk factors for CHD, blood glucose measures and the total plasma fatty acid content, in a young non-diabetic male population with angiographically proven CHD, and in an age and sex matched control group.

A2.1.2 Methods

A2.1.2.1 Study groups. The patient population consisted of the case-control groups described in Chapter 3.

A2.1.2.2 Anthropometric measures. Body weight corrected for stature, termed the body mass index ($BMI = \text{weight}/\text{height}^2$), was used to estimate overall body fatness.^{1083,1084} The scapular and triceps skinfold thickness were used to give an approximation of fat distribution.^{410,1084}

A number of measures of weight indexed to height have been developed to give an estimation of body fat.¹⁰⁸⁴⁻¹⁰⁸⁶ The BMI has become the most commonly used measure of fatness and reduces errors associated with height and weight tables.^{1085,1087} However, the BMI does not take into account differences in body composition,¹⁰⁸⁸ fat distribution³⁸³ or ethnic differences in heterogeneous populations.¹⁰⁸⁷ Despite these limitations, good correlations have been reported between body fat and weight when corrected for stature in females^{1087,1089} and males.^{1087,1090} In men the BMI appears to be the best easily obtainable anthropomorphic measure for estimating percent body fat in epidemiological studies.¹⁰⁸⁴ The BMI is a less reliable measure of body fat when applied to populations of different ethnic origins, different nutritional status, and different physical activity and physical fitness.^{1083,1084,1088} However, when employing the BMI for correlation with other indices for an individual, or for comparison between homogeneous groups, then comparison of the raw values can be made.¹⁰⁸⁴ Since this study involves relatively homogeneous male groups, the BMI has been utilised as a suitable estimate of overall body fatness.^{1083,1084,1088}

In adult males, scapular skinfold thickness gives the best correlation with percent body fat.¹⁰⁸⁴ There are clear limitations for the use of skinfold thickness measurements to estimate total body fat and the scapular and triceps skin fold thickness are used primarily to give an approximation of subcutaneous fat distribution.^{1083,1084,1091} Methodological limitations to the utilisation of skinfold thickness include variation in compressibility and fat content of subcutaneous tissues, and thickness of the skin,¹⁰⁹² an increasingly weaker correlation of skinfold thickness and other measures of body fat with age,¹⁰⁹³ accumulation of internal body fat and fat within muscle with aging, when subcutaneous fat is decreasing,¹⁰⁹⁴ and the compressibility of fat increasing¹⁰⁹⁵ (although this potential problem maybe over-estimated¹⁰⁹⁶). Individuals with the same triceps skinfold thickness and different arm circumference may differ as it takes more fat to cover a larger limb with a given thickness of subcutaneous fat.¹⁰⁹⁵ These potential problems can be limited by using an age and sex matched homogeneous study group with the measurement being performed by the one experienced observer.

A2.1.2.3 Glycosylated Hb. The method is described fully in Chapter 2.

A2.1.2.4 Plasma fatty acid analysis and lipoprotein measures. The methods are described in Appendix 1.

A2.1.2.5 Coronary angiography and CAD scoring. The methods are described in

Chapter 7.

A2.1.2.6 Statistical analysis. The methods are described in Chapter 2.

A2.1.3 Results

A2.1.3.1 Group comparisons. In the two study groups, there were no significant differences in body weight, height, BMI, current BP or triceps skinfold thickness. There was a very significant difference in scapular skinfold thickness between the two groups (Table A2.1). Differences in the major risk factors for CHD have been detailed in Chapter 3. The fasting blood glucose and HbA1C levels differed between the two groups. The blood glucose was lower in the control group (4.9 ± 0.08 vs 5.3 ± 0.10 mmol/L, $p=0.022$) and the HbA1C was also lower in the control group (7.3 ± 0.10 vs 7.8 ± 0.2 , $p=0.031$). The case group had a significantly greater percent of palmitic acid, and lower content of oleic, eicosenoic and docosapentaenoic acids in plasma than in the control group (see Appendix 1, Table A1.2).

A2.1.3.2 Anthropometric measures and CHD risk factors. In the control group, body weight had a significant positive correlation with triglyceride ($r=0.22$), HbA1C ($r=0.43$) and blood glucose levels ($r=0.39$), and a negative correlation with HDL cholesterol ($r=-0.30$) and apoprotein A1 levels ($r=-0.33$). There was no association with current systolic or diastolic BP, total cholesterol or apoprotein B levels. BMI was positively associated with triglyceride ($r=0.43$), blood glucose ($r=0.38$) and HbA1C levels ($r=0.41$) and systolic BP ($r=0.23$), and negatively associated with HDL cholesterol ($r=-0.26$) and apoprotein A1 levels ($r=-0.32$). There was no relationship with total cholesterol and apoprotein B levels. Triceps skinfold thickness was not related to any of the risk factors evaluated. Scapular skinfold thickness had a borderline relationship with the triglyceride ($r=0.27$), HDL cholesterol ($r=-0.26$), apoprotein A1 ($r=-0.39$) and apoprotein B ($r=0.36$) levels.

In the case group, body weight was related directly to blood glucose levels ($r=0.32$). No consistent relationship between body weight or BMI, and blood lipoprotein concentrations was present. There was a positive relationship between BMI and HbA1C levels ($r=0.31$) and blood glucose levels ($r=0.45$), and diastolic BP ($r=0.24$). Again, triceps skinfold thickness was unrelated to any variable. However, scapular skinfold thickness was directly related to diastolic BP ($r=0.24$), HbA1C ($r=0.25$) and blood glucose levels ($r=0.45$), and negatively related to the HDL cholesterol level in the group with CHD ($r=-0.29$).

In summary, in the control group, relationships between BMI, body weight and scapular skinfold thickness, and specific lipoproteins, measures of increased blood glucose levels and systolic BP were present. These findings demonstrate distinct differences between the case and control groups. In particular, a positive association between a measure of central obesity and measures of blood glucose levels was evident only in the case group. No relationship was found between the anthropomorphic measures and age, previous cigarette intake and alcohol intake.

A2.1.3.3 Anthropometric measures and plasma fatty acids. In the case group, body weight had a negative but borderline association with the percent of plasma

myristic ($r=-0.24$), palmitic ($r=-0.21$) and arachidonic acids ($r=-0.21$). Scapular skinfold thickness was significantly and inversely related to the percent of plasma myristic ($r=-0.35$) and palmitic acids ($r=-0.36$). Oleic acid content was positively associated with body weight ($r=0.35$), BMI ($r=0.27$), scapular ($r=0.47$) and triceps skinfold thickness ($r=0.36$) only in the case group.

These relationships differed in the control group. The plasma content of myristic ($r=-0.24$) and palmitic acids ($r=-0.26$) were negatively associated with body weight. The percent of alpha-linolenic ($r=0.24$), dihomo- γ -linolenic ($r=0.42$), arachidonic ($r=0.44$) and stearic acids ($r=0.32$) had a positive association with body weight. BMI correlated with the percent of plasma stearic ($r=0.32$), eicosadienoic ($r=0.26$), dihomo- γ -linolenic ($r=0.53$) and arachidonic acids ($r=0.51$). There was no consistent relationship with the skinfold thickness measures in the control group.

A2.1.3.4 Glucose measures and CHD risk factors. In the control group, the HbA1C level correlated positively with triglyceride ($r=0.25$) and apoprotein B levels ($r=0.28$), and inversely with the HDL cholesterol level ($r=-0.25$). The blood glucose level correlated positively only with the triglyceride level ($r=0.25$). In comparison, the blood glucose measures in the case group were unrelated to any lipoproteins. Since the possibility of a metabolic relationship between glucose metabolism and plasma fatty acid exists, the association between these factors and both blood glucose measures was evaluated. In the control group there was a positive association between percent dihomo- γ -linolenic ($r=0.24$), arachidonic ($r=0.34$) and alpha-linolenic acids ($r=0.27$) and HbA1C level, and a negative association between percent plasma oleic acid ($r=-0.29$) and HbA1C levels. These associations were not observed in the case group.

A2.1.3.5 Multivariate analysis with HbA1C as the dependent variable. Using multivariate analysis, with HbA1C as the dependent variable and the associated plasma fatty acid, lipoproteins, blood glucose level and anthropomorphic measures as the independent variables, HbA1C was predicted negatively by oleic acid and positively by alpha-linolenic acid values in the control group. In the case group only BMI retained a relationship with HbA1C levels (Table A2.2).

A2.1.3.6 Correlations with CAD Scores. The relationship between the extent and severity of CAD and plasma lipoproteins, the plasma fatty acid content and the anthropomorphic measures was evaluated initially by linear correlation. No relationship existed in the case group between any of the coronary artery scores and BMI, body weight, height, triceps skinfold thickness or the blood glucose level. HbA1C levels had a weak but significant linear correlation with both the severity (stenosis score) and extent (atheromatous score) of CAD (Table A2.3). Scapular skinfold thickness correlated with the extent but not the severity of CAD (Table A2.3). In addition, CAD severity linearly correlated with age ($r=0.25$), and the extent of CAD correlated with age ($r=0.27$), cholesterol ($r=0.23$) and apoprotein B levels ($r=0.39$). The number of normal segments correlated negatively with age ($r=-0.23$), cholesterol ($r=-0.33$) and apoprotein B levels ($r=-0.39$). The percent of plasma myristic acid was positively associated with measures of both extent and severity of CAD, and negatively with the number of normal segments (Table A2.4). Oleic acid values correlated positively with the number of normal segments, and

aracidonic acid values correlated with the extent of CAD (Table A2.4).

A2.1.3.7 Multivariate analysis with coronary artery scores as the independent variables. In order to further evaluate these relationships, multivariate regression analysis was undertaken using the angiographic scores as dependent variables. Those plasma fatty acid with a linear relationship to one or more angiographic scores (myristic, oleic and arachidonic acids), as well as diastolic BP, HDL cholesterol and HbA1C levels and scapular skinfold thickness were entered as independent variables, in consideration of the possible interrelationships between CAD, hyperglycaemia, insulin resistance, central body fat and fatty acid. Myristic acid content of plasma and scapular skinfold thickness remained significantly related to the extent of CAD. Of the variables examined, only myristic acid content remained independently related to the severity of CAD (Table A2.5). BMI and scapular skinfold thickness were entered separately and the variance for the extent of CAD was found to be contributed to by the percent of plasma myristic acid and scapular skinfold thickness (Table A2.5). The severity of CAD was predicted only by the percent of plasma myristic acid, while the number of normal segments of coronary arteries was predicted negatively by the apoprotein B level and the percent of plasma myristic acid (Table A2.5).

A2.1.4 Discussion

The results of this study provide some unique observations not previously reported. An association between specific fatty acid, HbA1C values and scapular skinfold thickness was observed, but only in subjects with CHD, and not in a normal control group. The control group differed further, in that associations were found between HbA1C and blood lipoprotein levels and specific plasma fatty acid, which were not found in the group with premature CHD. Moreover, convincing relationships between a measure of central body fat (scapular skinfold thickness), and a plasma fatty acid (myristic acid), with CAD have been defined. A major implication from these findings is that the relationship between central body obesity, CHD risk factors and abnormal glucose metabolism may be due to abnormalities of fatty acid metabolism in those individuals developing CHD.

A2.1.4.1 Central body obesity, CHD risk factors and CHD. Many of the findings in this study are consistent with the current literature in which associations between central distribution of body fat and metabolic abnormalities are well described.^{642,383,386} Body weight and BMI cannot be considered clinically significant risk factors for CHD in our study group. In contrast, increased central adiposity, as measured by scapular skinfold thickness, was found to be a measurable risk marker for CHD. The relationship with CHD is strengthened by the fact that an independent association between scapular skinfold thickness and the extent of angiographically defined CAD was maintained in multivariate analysis. These observations are supported by a recent angiographic case-control study which demonstrated an association between android adipose tissue distribution in females and coronary atherosclerosis.¹⁰⁶¹ Males with excess deposition of fat in the abdominal region were also likely to experience increased risk of CHD.¹⁰⁶¹

In this study, associations between blood glucose measures and scapular skinfold

thickness were found only in the case group. It needs to be emphasised that this association was present in non-diabetic subjects with a modest but nevertheless, significantly higher fasting blood glucose and HbA1C levels than the matched normal group.

The CHD group differed considerably from the control group in the relationships observed between scapular skinfold thickness and plasma lipoproteins. Increasing scapular skinfold thickness was associated with a decreased HDL cholesterol level, and increased triglyceride and apoprotein B levels in the control group. Apart from an association with the HDL cholesterol level, these associations were not observed in the CHD group.

The clustering of a relationship between diastolic BP, fasting blood glucose, HbA1C and HDL cholesterol levels and scapular skinfold thickness in the case group is consistent with the current literature, and with prevailing concepts regarding regional body fat distribution and its relation to CHD.^{383,749,1051,1058,1059,1066,1069,1097} Moreover, the observations concerning fasting blood glucose and HbA1C levels and scapular skinfold thickness are also consistent with the known relationship between glucose metabolism and regional fat distribution.¹⁰⁶⁹

A2.1.4.2 Blood glucose, HbA1C concentrations and CHD in the non-diabetic.

The utilisation of HbA1C measurements to estimate medium term blood glucose levels has been well defined in individuals with diabetes mellitus and glucose intolerance.^{366,1098} A similar graded relationship with blood glucose may occur in non-diabetics.¹⁰⁹⁹ In a large non-diabetic adult population, HbA1C values were found to be normally distributed, were unrelated to age and correlated with fasting plasma glucose in both men and women, and with obesity in women.¹¹⁰⁰ In the same population, after adjusting for age and obesity, HbA1C levels were significantly associated with plasma cholesterol, LDL cholesterol, total plasma triglyceride and VLDL cholesterol levels in men.¹¹⁰⁰ In another study of a normal but heterogeneous male group, Hb glycosylation was found to be directly proportional to levels of total and LDL cholesterol, and indirectly to HDL cholesterol levels.¹⁰⁹⁹ Glycosylated Hb levels correlated significantly with fasting plasma glucose levels.¹⁰⁹⁹ Our observations are similar, in that, in our normal population group, HbA1C levels had a significant positive relation with triglyceride and apoprotein B levels, and negative association with the HDL cholesterol level.

The results of this study point to a difference in glucose homeostasis in the two groups. Even though neither group included diabetics, the CHD group had significantly higher blood glucose and HbA1C levels. Higher blood glucose levels, which are not at the level of diabetic hyperglycaemia, may be associated with CHD.¹⁰⁹⁷ This association is particularly strong after a glucose load.^{1101,1102} However, the relationship between a casual blood glucose level and CHD in non-diabetic males is more controversial, with some studies supporting an association,¹¹⁰³ while others do not.¹⁰⁹⁷ The association appears to be more consistent in women.¹⁰⁹⁷

The negative relationship between HDL cholesterol and HbA1C levels observed in this study may relate to the fact that insulin resistance can reduce plasma clearance of triglycerides due to impaired activity of lipoprotein lipase, which may further

increase VLDL levels and reduce HDL cholesterol levels.⁶³⁵ Therefore, a reduced HDL cholesterol could be associated with direct or indirect measures of insulin resistance, as is observed in diabetics,¹¹⁰⁴ and as found in the normal control group in the present study and as reported by others.⁷⁴⁹

A2.1.4.3 Fatty acids and body fat distribution. The mechanisms of the metabolic and/or genetic relationships between the various risk factors associated with central body fatness, including insulin resistance, are at present only speculative.³⁸⁵ However, it is possible that the relationships may be due to the mobilisation of specific fatty acid from abdominal fat.^{383,385} Portal adipose tissue is sensitive to mobilisation of free fatty acid due to a preponderance of beta-adrenergic receptors with little alpha-adrenergic inhibition.³⁸⁵ Therefore, abdominally obese individuals may have a higher portal free fatty acid concentration when mobilisation is stimulated by such factors as stress, anger, frustration and smoking.³⁸⁵ The increased lipolysis of portal adipose cells in men and post-menopausal women may contribute to abnormal hepatic metabolism due to high portal free fatty acid concentration. Portal free fatty acids stimulate gluconeogenesis and inhibit the hepatic binding of insulin,^{385,1082} processes which are dependent on fatty acid oxidation.³⁸⁵ Both hyperinsulinaemia and increased peripheral release of free fatty acid resulting from insulin resistance can increase hepatic synthesis and secretion of triglycerides, leading to increased levels of VLDL⁶³⁵ and an increase in the metabolic products of VLDL, including apoprotein B, LDL and remnant VLDL particles in peripheral blood.^{385,386} In addition, or alternatively, excess portal free fatty acid release may contribute to atherogenesis by influencing the fatty acid content of circulating lipoproteins and/or the generation of other powerful risk factors.³⁸⁵

Because of this potential mechanism, we examined the relationships between plasma fatty acid and the various risk factors associated with central body fatness. An association was demonstrated between three plasma fatty acids (myristic, palmitic and oleic acids) and measures of body fat. Other fatty acid (docosapentaenoic and oleic acids) were associated with HbA1C and fasting blood glucose levels respectively. In addition, given the difference in glucose homeostasis and/or protein glycosylation in males with premature CHD compared to normals, the relationship between fatty acid and measures of blood glucose was examined. Support for such an evaluation comes from an angiographic study of young males with CHD, using multivariate analysis, which established a direct association between angiographic severity of CAD and a positive oral glucose tolerance test, and levels of palmitic and arachidonic acids in adipose tissue and in blood triglycerides.¹¹⁰⁵ Although the measurement of fatty acids differed from this present study, the findings did implicate differences in body content of plasma fatty acid and abnormal carbohydrate homeostasis as significant risk factors in males with angiographically proven premature CHD.¹¹⁰⁵ In the same study no association was found between disease severity and triceps and abdominal skinfold thickness, while scapular skinfold thickness was not measured.¹¹⁰⁵

A2.1.4.4 Possible explanation of results. The absence of an association between HbA1C and blood glucose levels and diastolic BP with central body fat in the control group, and the presence of this association in the case group in the present study, supports the possibility of an aetiological relationship between altered glucose homeostasis, hypertension, central obesity, plasma fatty acid and abnormal lipopro-

The glycosylation process can affect almost all extracellular proteins including plasma lipoproteins.^{1099,1106-1108} Therefore any association between HbA1C and risk factors for CHD, and CHD itself, may well be attributed to glycosylation of proteins. The potential significance of the glycosylation of LDL is emphasised by the observation that glycated LDL shows an affinity for the apoprotein B receptor in human fibroblasts,¹¹⁰⁷ and that the catabolism of glycated LDL in guinea pigs is diminished.^{136,1099} The possible interference to lipoprotein metabolism is further highlighted by the decreased ability of glycated LDL to inhibit intracellular hydroxymethyl glutaryl coenzyme A reductase activity.¹¹⁰⁷ and the fact that glycated LDL does not increase cholesteryl ester synthesis which normally occurs with endocytosis of LDL.¹⁰⁹⁹ Furthermore, under experimental conditions, glycated LDL is more prone to lipid peroxidation, which has emerged as an important factor in atherogenesis.^{1109,1110}

Another interpretation of the present results is that glycosylation of plasma proteins including LDL, may be influenced by differences in the composition and/or levels of plasma fatty acid. Alternatively, the concentrations and content of fatty acid in plasma may influence insulin effectiveness, increasing resistance to the action of insulin. The outcome of such metabolic events would be to prolong the presence of atherogenic LDL particles in the circulation. Therefore, the process of glycosylation of LDL may contribute to the increase in plasma LDL concentration, and as a result, the increased cholesterol levels observed in hyperglycemic diabetic and non-diabetic subjects.¹⁰⁹⁹ The measurement of a glycosylated protein, namely HbA1C, may in fact only provide an indirect measure of glycosylated LDL, and hence an association between HbA1C and CHD.

A2.1.5 Summary

1. The distribution of subcutaneous fat, as measured by triceps and scapular skinfold thickness, appears to differ between young males with and without CHD who have the same overall body mass measurement. In addition, young non-diabetic males with CHD have increased measures of glycosylation of plasma proteins, viz HbA1C.

2. An association between a measure of central obesity and measures of blood glucose and HDL cholesterol level are observed only in the group with CHD. In the same group, the glycosylation appears to be predicted more by the plasma content of C18 group of fatty acids.

3. The extent of angiographically defined CAD is better predicted by the scapular skinfold thickness and myristic acid than other risk factors for CHD which are associated with the measure of central body obesity.

4. The combination of abnormal glucose homeostasis, truncal obesity and lipoprotein abnormalities impart significant risk for developing CHD. We have demonstrated that plasma fatty acid content may play a role in this relationship.

5. The interactive relationships between glucose metabolism, fatty acid metabolism, body fat distribution and lipoprotein abnormalities illustrates that a potential relationship with this group may exist with platelet function. The possible confounding influence of these relationships on the main results of the case-control study needs to be appropriately assessed in the final analysis.

Table A2.1. Anthropometric characteristics of the case and control groups.

	Controls	Cases	
BMI (kg/cm ² 10-5)	257 +/-5	266 +/-5	NS
Triceps (cm)	11.7+/-0.8	12.5+/-0.7	NS
SSFT (cm)	13.8+/-0.7	17.1+/-0.9	p=0.006
Height (cm)	176.0+/-1.2	175.0+/-1.1	NS
Weight (kg)	80.0+/-1.7	80.8+/-1.5	NS

SSFT=scapular skinfold thickness, triceps=triceps skinfold thickness. Results shown are mean+/-standard error of the mean. NS=no significant difference.

Table A2.2. Multiple regression analysis with HbA1C as the dependent variable and plasma lipoproteins, fatty acids, and anthropometric measures as the independent variables.

Dependent Variable	Independent Variable	B	SE B	Rsqr	Sig
HbA1C (Cases)	BMI	0.1695	0.0720	0.1552	0.0254
HbA1C (Controls)	C18:1n9	-0.0105	0.0041	0.1817	0.0185
	C18:3n6	0.0622	0.0275	0.3425	0.0341

B=regression coefficient, Rsqr=coefficient of determination. SE=standard error, sig=p value. See Table 9.3 for fatty acid abbreviations.

Table A2.3. Linear relationships between CAD scores and scapula skinfold thickness, HbA1C and fasting blood glucose levels in the case group.

Variable	Scapular	HbA1C	BSL
CAS	0.38#	0.31#	0.15
CSS	0.15	0.27*	0.22
mCAS	0.38#	0.30*	0.13
mCSS	0.10	0.25*	0.19
Normseg	-0.12	-0.04	-0.01

*=P<0.05, #=P<0.01,

Normseg=number of normal segments, CAS=coronary atheromatous score, mCAS=mean CAS, CSS=coronary stenosis score, mCSS=mean CSS, BSL=blood glucose level, scapular=scapular skinfold thickness, HbA1C=glycosylated Hb.

Table A2.4. Linear correlations between CAD scores and plasma fatty acids.

Variable	C14:0	C18:1n9	C20:4n6
Normal	-0.35#	0.30*	0.05
CAS	0.32#	-0.13	-0.24*
mCAS	0.33#	-0.14	-0.23*
CSS	0.34#	-0.13	-0.18
mCSS	0.36#	-0.16	-0.17

##P<0.001, #=P<0.01, *P<0.05..ls1

See Tables 9.3 & 9.5 for abbreviations.

Table A2.5. Multiple regression analysis with CAD scores as the dependent variable and fatty acids and CHD risk factors linearly related to angiographic scores, including HbA1C and HDL cholesterol levels, diastolic BP and scapular skinfold thickness, as independent variables.

Dependent Variable	Independent Variable	B	SE B	Rsqr	Sig
CAS	Scapula	0.08808	0.02860	0.1382	0.0043
	C14:0	0.03054	0.01201	0.2868	0.0162
mCAS	Scapula	0.07098	0.02343	0.1385	0.0049
	C14:0	0.02384	0.00984	0.2756	0.0215
CSS	C14:0	0.04366	0.02043	0.1249	0.0403
mCSS	C14:0	0.03854	0.01762	0.1300	0.0362
NORM	Apo B	-0.0939	0.0263	0.1959	0.0014
	C14:0	-0.0090	0.0033	0.3316	0.0104

See Tables A2.2, A2.3 and A2.4 for abbreviations

APPENDIX 3

BEHAVIOURAL PSYCHOLOGICAL RISK FACTORS IN CORONARY HEART DISEASE

A3.1 BEHAVIOURAL AND PSYCHOLOGICAL ATTRIBUTES AS RISK FACTORS FOR CORONARY HEART DISEASE

A3.1.1 Introduction

A diverse variety of behavioural and psychosocial variables that may characterise individuals prone to cardiovascular disease have been identified. These include the type A behavioural pattern,¹¹¹¹⁻¹¹¹⁴ life dissatisfaction,¹¹¹⁵ depression,^{1115,1116} self abasement,¹¹¹⁵ anger,^{1112,1117} perceived chronic life struggle,¹¹²² status incongruity,¹¹¹⁵ self involvement,^{1121,1122} inadequate social network,¹¹²³ and hostility¹¹¹⁸⁻¹¹²⁰ and the list goes on.¹¹¹⁵

There are pathophysiological models which provide a potential biological basis for the increased risk associated with behavioural and psychosocial variables,^{376,1124-1128} An example relevant to this Thesis is an enhanced neuroendocrine system resulting in an excessive excretion of adrenaline in response to external/internal stimuli in type A cynical individuals.¹¹²⁵ The potential for physiological levels of adrenaline to affect platelet function has been demonstrated.^{71,72} Therefore, possible confounding factors responsible for indirectly producing the observed difference in platelet function may be differences in behavioural factors which influence neuroendocrine responses. This section, therefore, considers some of the major postulated behavioural and psychological characteristics in the study groups, firstly to determine if differences exist and secondly to determine if there is any relationship with angiographic CAD.

A3.1.2 Type A Behaviour and CHD

Type A behaviour is a global constant which is applied to individuals who possess many of the defining characteristics, although not necessarily all.¹¹²⁹ Retrospective and prospective epidemiological research has linked the global type A behaviour pattern with clinical manifestations of CAD, including initial incidence and recurrence of MI.^{203,1115} A structured interview (SI) method, separating persons with either a type A or type B behaviour pattern, appeared to predict the development of CHD independent of the known CHD risk factors.²⁰³ Similar findings were reported from a sub-group of the Framingham study.¹¹³⁰ Indeed, the association of CHD with type A behaviour appeared to occur also in women when using the specific method of assessment chosen by the investigators in the Framingham Study.¹¹³⁰ An additional form of differentiating type A and B behaviour patterns, the Jenkins Activity Survey (JAS) was also found to predict CHD events (angina pectoris and Q-wave infarctions combined).¹¹³¹ Variation in the rates of progression of CAD for individuals with type A behaviour, compared with type B behaviours, suggests that behavioural factors may play a role post priori in the development of CAD.¹¹³²

Overall, current evidence does not support the theory that type A behaviour contributes directly to atherosclerosis lesion formation.⁹⁸⁵ More recent studies have failed to corroborate the initial observations of an association between type A behaviour and CHD. The studies include those from the general population,¹¹³³⁻¹¹³⁶ persons with established CHD,¹¹³⁷⁻¹¹³⁹ and angiographic studies.^{1121,1140} A number of possibilities may explain these divergent results, including variation in sampling and measure-

ment, differences in the population groups, qualitative changes in the nature of the type A behaviour over time, and the publicity given to the concept. One of the potential explanations most explored has been that type A behaviour, as measured by current methods, may simply be a diluted marker for other behavioural characteristics that carry the risk for CHD.¹¹⁴¹

The type A classification is based on a preponderance of type A characteristics which were initially determined by the structured interview method developed by Friedman and Rosenman.^{1142,1143} A factor analysis of the variables specified in the structured interview identified more specific components of the type A behaviour pattern that appeared to discriminate between cases of CHD and controls.¹¹⁴³ "Competitive drive" and "impatience" were the most discriminating components. The elements of competitive drive that distinguished the groups were "explosive voice modulation", "potential for hostility" and "vigorous answers to questions".¹¹⁴³ Self-reports of anger were also discriminatory. This analysis thus provided evidence for a possible association between CHD and anger-hostility as the component of type A behaviour engendering the CHD risk. In fact, four of the seven significant components were directly related to anger, and the other three could be motivated by anger. Alternatively, of the benign and coronary prone facets of the type A behaviour pattern, hostility may best exemplify the latter.¹¹⁴⁴

A3.1.3 Hostility and CHD

Delineation of a type A personality by the JAS and/or SI method is reported to be predictive of future CHD.^{203,1131,1145} As discussed, the main components of the type A personality profile predictive of CHD may be measures of anger and hostility.^{1146,1147} Indeed, independent measures of hostility also have predictive capabilities for the development of CHD in prospective studies,^{1118,1124,1147} supporting the concept that the hostile and cynical type A individuals are the "at risk" group within the coronary prone behaviour profile.¹¹²⁵

An hostility complex, comprising interrelated hostility and anger constructs and proposed as the pathogenic component of the type A behaviour,¹¹⁴³ has been refined.³⁷⁸ The anger-hostility dimension is the dominant characteristic among the coronary-prone type A behaviours.¹¹²⁸ The relationship of the type A anger dimension to the incidence of CHD is also present in the Framingham Study.¹¹³⁰

The hostility-anger items and scales of the psychometric measures used to assess Type A behaviour which have been found to discriminate type A/B behaviours,^{1128,1148} also correlate with enhanced cardiovascular responsiveness of type A subjects.¹¹²⁷ Hostility, accompanied by self-reported low anxiety, may be linked to specific clinical syndromes of coronary disease.¹¹⁴⁹ Angiographic studies have also show a correlation between hostility/anger constructs and the severity of angiographically defined CAD.^{1147,1150}

In summary, there appears to be reasonable evidence that the anger/hostility, and therefore this dimension of the type A behaviour, may confer an increased risk for CHD.^{377,1128} The evidence, however, still remains controversial and has not been fully validated.^{1128,1141}

A3.1.3.1 What Measure of Hostility Indicates the Risk for CHD? The Minnesota Multiphase Personality Inventory (MMPI) hostility score (Ho score) has gained considerable support as a risk factor for CHD.^{1118,1147,1151} Corroboration of the potential relationship between CHD and hostility is provided by a similar relationship being demonstrated with other measures of hostility.^{1152,1153} Another study only reports an association in groups who already have CHD or hypertension, and not in healthy population groups.¹¹²⁰ Of additional interest is that the MMPI Ho score may be associated with factors having broad effects on overall survival as well as the development of CHD.¹¹¹⁸

Nevertheless, any relationship between CHD and the various hostility measures remains tentative. A number of more recent studies report conflicting results, both for measures using the MMPI Ho score¹¹⁵⁴⁻¹¹⁵⁶ or alternative measures of hostility.¹¹⁴⁴ Refinement of a measure of hostility has shown that a hostility measure which correlates with indices of neuroticism, as compared to a hostility measure unrelated to neurotic tendencies, is inversely related to CHD.¹¹¹⁹ The overall hostility score used in the study was unrelated to CHD.¹¹¹⁹

A3.1.3.2 Hostility and CAD Defined Angiographically. Gender, the MMPI Ho score of the MMPI and the behavioural type classification (A or B) are the strongest independent predictors of the degree of coronary atherosclerosis in an early angiographic study.¹¹⁴⁷ The MMPI items measuring chronic hate and anger are the most significant discriminators.¹¹⁴⁷ More recently the potential for hostility was shown to be associated with severity of atherosclerosis for patients who suppress anger.^{1152,1153}

Diverse factors may be pertinent to the association between CAD and behavioural factors, and anger, hostility and aggression are prominent in this regard. Unquestionably, evidence of an association between an anger and/or hostility construct and CAD has been increasing.¹¹⁵⁷ Nevertheless, further evaluation is required to delineate the association, if any, as it is apparent that a distinction between the intensity of the experience of anger, the anger as a personality trait, how anger is expressed, and how frequently it is experienced may be necessary.^{1129,1152,1153,1157} Moreover, the association with hostility may be through other factors which are measured in the constructs used to assess hostility.^{1119,1158} The psychological trait of cynicism¹¹⁵⁸ or the specific feature of non-neurotic hostility¹¹¹⁹ may be more closely related to CAD.

A3.1.4 Potential Mechanisms of Behavioural Factors in CAD

Acute autonomic nervous system changes and neuroendocrine effects³⁷⁶ may provide a link between behavioural and psychosocial attributes and the development of CAD.³⁷⁶ It is hypothesized that recurrent episodes of acute "psychophysiologic" reactivity may promote atherogenesis, either through the haemodynamic disturbances related to acute rises in heart rate and BP (increasing turbulence and sheer stress resulting in increased risk of plaque fissuring) and/or due to increased release of certain neuroendocrine hormones.³⁷⁶

Individuals vary greatly in the magnitude of their physiologic response to behavioural stimuli, but any associations with the pathogenesis of CHD remain in the realm of

research.¹¹²⁶ Type A individuals often exhibit more pronounced cardiovascular and neuroendocrine responses (eg. the enhanced release of catecholamines) than Type B individuals when challenged by frustrating laborious tasks or other behavioural stimuli.^{1111,1127,1128} Patients with CHD may exhibit greater pressor reactions to behavioural stressors than non CHD patients or controls.¹¹⁵⁹ Aggressive and hostile individuals appear to secrete more noradrenaline compared to more passive or anxious subjects.¹¹⁴² Type A subjects exhibit greater muscle vasodilation and increases in noradrenaline, adrenaline and cortisol during mental work, and a greater increase in testosterone during reaction time performance compared to type B subjects.¹¹²⁷ Male type A subjects generally respond to overstimulation with a higher adrenaline response.¹¹²⁸ Resting levels do not appear to differentiate between type A and type B subjects, or persons with CHD.¹¹²⁴

Such changes in the neuroendocrine system may indeed promote atherogenesis by a number of possible avenues. For example, haemodynamic disturbance secondary to an increase in heart rate and/or an increase in BP may lead to greater turbulence and sheer stress on the arterial wall, factors which probably play a role in the pathogenesis of CAD.^{42,1160,1161} Furthermore, the increased release of hormones, such as adrenaline, and the effect of these catecholamines on platelet aggregation provide a potential pathophysiological mechanism in the atherogenic process.^{71,72} Observations such as these provide a stimulus for further research.

A3.1.5 Methodological Limitations in Published Studies

It is clear from the above discussion that not only are the associations between CAD and behavioural or psychosocial characteristics of uncertain significance, but also that the measures utilised to evaluate the possible relationships have major limitations. In this regard, specific caveats need to be mentioned regarding the methods used elsewhere in this study.

(i) *Generality and cross-cultural validity of type A measures:* Both the SI and JAS have been used in studies in many countries and have been found to be predictive of angina. The information at present is insufficient for women, rural populations, changes associated with aging and for non-industrialized societies.¹¹²⁴

(ii) *Limitations inherent in the type of instrument:* Personality inventories are dependent on self-insight, candid-response, response bias, the exclusively verbal/symbolic and abstract level of description and the absence of a varied situational context. Since the JAS was developed as an alternative to the SI, the SI is a more valid instrument for the measurements defining type A behaviour, albeit more costly and more difficult to implement.¹¹²⁴

(iii) *Limited utility of the structured interview:*¹¹²⁴ The SI is allegedly a more powerful predictor of CHD incidence than the JAS in a population consisting of equal percentages of type A and type B persons.¹¹³² However in the SI, with samples having a high percentage of type A personalities, global interview assessments may lose their predictive power due to limited variability in the measurement. Furthermore, in a sample of patients with manifest CHD, stylistic behaviour may be altered by medications and also from the psychosocial effects resulting from the

publicity regarding type A behaviour. Finally, subjects may consciously or unconsciously temper stylistic mannerisms in artificial, or what they see as contrived circumstances.

(iv) *Dilution effect of risk estimation by the presence of CHD*: A potential problem for identifying behavioural and psychosocial risk factors with regard to the effect on outcome, is the influence of the underlying disease itself on prognosis.³¹⁹ This potential confounding factor has been proposed as a cause of previous difficulties in establishing increased cholesterol as an important prognostic predictor in patients with established CHD.³¹⁹ Therefore, some risk factors may be good predictors of disease in non-CHD populations, but a poor predictor in populations with established disease, the disease itself mainly determining the frequency of future cardiac related events. As previously discussed, studies exist showing that type A is predictive for the development of CHD.^{203,1130,1143} In groups with CHD, these measures have not been consistently discriminative, and therefore reliability has been questioned, as well as their validity.

The nature, and even possibly the existence, of any relationship between behavioural factors and CAD is at present a matter of scientific enquiry rather than an established body of evidence establishing a direct or causal association.

A3.1.6 Methods

A3.1.6.1 Introduction. Research findings based on interviews are not uniformly successful due to bias arising from subjective interpretations.¹¹⁶² More objective methods of encoding and quantifying information are by self report scales, and self report behavioural rating scales.¹¹⁶² Although bias due to "halo effects", and leniency and proximity errors are difficult to control for in the latter methods, the interview method is highly dependent on the skills, training and experience of the interviewer, and the responsiveness of the subject.¹¹⁵⁷ With due regard for the limitations of both methodologies, self report rating scales were selected as most appropriate for the present study and are detailed below.

A3.1.6.2 Measurement of Type A Behaviour Pattern. The initial method of measuring the type A behaviour pattern was by the SI, as defined by Rosenman and Friedman.^{1142,1163} The SI measures the style and manner of delivery of the information during an interview by means of numerically graded characteristics. Another method, the JAS, measures the content of type A characteristics from a self-administered questionnaire, and a graded risk is then assigned.^{1145,1164} The Framingham type A scale also measures the content of type A characteristics from a self-administered questionnaire.^{1130,1165,1166} Each method appears to measure some factor, or factors, unique to its own design, with a significant element of overlap.^{1164,1167,1168} The Western Collaborative Group Study provides evidence of a "dose-response" curve for the JAS,^{1164,1167} whereas the SI and the Framingham type A scale did not attempt to show such a graded response relationship.¹¹³⁰ However, the SI and the JAS type A patterns are not identical, having an agreement of about 68% overall.^{1164,1167} The common aspects of these methods have been delineated as self-reported pressure drive, hostility, competitiveness and energy level.¹¹⁴³

A3.1.6.3 Jenkins Activity Survey. This self report questionnaire has previously been shown to validly identify males having the Type A behaviour pattern with over 72% accuracy.¹¹⁴⁵ However, self report measures of the global Type A have consistently been found to be more weakly related to CHD endpoints than the SI-based assessment.³⁷⁷ With this in mind, and in view of the emergence of a hostility/anger complex as the possible "toxic" component of Type A,³⁷⁸ it is important to understand what is being measured by the JAS. Self report measures of the Type A behaviour pattern assess only parts of the overall behaviour pattern, and this varies from one instrument to another.¹¹⁶⁹ Independent factor analysis on the JAS items shows that it is measuring 3 major and conceptually independent behavioral syndromes which are stable over time, viz: (i) hard driving, (ii) job involvement, and (iii) speed and impatience.¹¹⁷⁰ None of these however, directly assess hostility. This method therefore provides a measure of the global Type A behaviour pattern, which does not have a strong independent hostility component. Furthermore, the training requirements for individuals conducting the SI are appropriately strict and inexperience leads to invalid interpretation.¹¹⁶⁹ For these reasons the JAS was selected for measurement of the global Type A pattern, with cognizance of the limitations associated with the method.

A3.1.6.4 Hostility. The hostility scale (Ho score) from the MMPI has previously been validated.¹¹⁷¹ However, there is the probability that this self-report questionnaire measures a general mistrust of human nature, or cynicism,^{1172,1173} rather than true hostility. However, importantly, the construct(s) that are being measured by this scale are remarkably stable over time.¹¹⁵¹ This reproducibility over time enhances the usefulness of the method for epidemiological and investigational purposes, even though the exact construct being measured may need redefining in future.

Numerous alternative measures of hostility do exist, such as the Buss-Durkee Hostility Inventory (BDHI).¹¹⁵⁷ However, for this latter measure, factor analyses do not provide empirical support for the validity of the BDHI sub-scales as measures of the types of hostility originally proposed by the investigators.¹¹⁵⁷

A3.1.6.5 Anger. A Multidimensional Anger Inventory (MAI) has been previously validated for use in "Westernised" populations.¹¹¹⁷ This inventory provides a multidimensional assessment of anger, which includes a subset measure of hostility and suppressed anger.¹¹¹⁷ The MAI was constructed to contain items measuring specific dimensions of anger, namely the frequency, duration, magnitude, the presence of a hostile outlook, the range of anger producing situations and the mode of expression.¹¹¹⁷ Specific items of the MAI were used to measure the hostile outlook (MAI hostility score) and suppressed anger (anger-in) for this study, as well as the overall score (MAI score).

A3.1.6.6 Study groups evaluated. Three study groups were evaluated. The older CHD group is described in section 7.2, and the younger CHD group and the young control group are described in Chapter 3. *The older and younger groups were considered together because of the strong potential to develop psychological and behavioural symptoms after a longer period of having the knowledge of CHD.* For example, older patients usually have more symptoms because of more severe disease, and therefore changes in behavioural measures may occur because of these

symptoms resulting in a systematic measurement bias.

A3.1.7 Results

A3.1.7.1 Comparisons between study groups. With regard to the behavioural measures, there were no significant differences between the older and younger CHD groups except for the MMPI Ho measurement. The young normal control group had a significantly lower MMPI Ho score than either of the two CHD groups (Table A3.1). No other score was different between the control and the two CHD groups, although there was a lower JAS in the young normal control group, but this did not attain statistical significance with this study sample (Table A3.1).

A3.1.7.2 Correlations with the psychological measures. There was no relationship between the NYHA classification for functional class of angina or the number of MI with any of the psychological measures for the young CHD group (mean age 44.6 years). The MMPI Ho score positively correlated with the volume of the previous cigarette consumption, alcohol intake, diastolic BP, BMI, and cholesterol and HbA1C levels (Table A3.2). There were only sporadic associations between the other psychological measures and risk factors for CHD (Table A3.2). The MMPI Ho score also correlated with the angiographic severity score and was of borderline significance in its relation with the coronary atheromatous score (Table A3.3). The MMPI Ho score also had an inverse association with the number of normal segments (Table A3.3). Predictably, there was a strong association between the MMPI Ho score and the MAI hostility score, less so with the MAI score and the MAI anger-in score, and the least association with the JAS (Table A3.4).

In the older CHD group with a broad age range (mean 53.2 years), the MMPI Ho score correlated ($p < 0.05$) with age and NYHA functional class for angina, associations which were not significant for the younger group. The associations between the risk factors for CHD and hostility were not as many or as strong in the older group when compared with the younger CHD group, and only included the BMI and the cholesterol level (Table A3.5).

There were no associations of significance between any of the risk factors and the psychological measures in the control group without CHD.

More strongly positive and more consistent univariate correlations were observed with angiographically defined CAD in the older CHD group than in the younger group. The MMPI Ho for score correlated with the number of vessels having haemodynamically significant lesions, with the severity of CAD, with the extent of CAD and inversely with the number of normal segments (Table A3.6). The strongest correlation was obtained with the severity of CAD, as it was in the young CHD group.

Similar to the young CHD group, there was a strong association between the MMPI Ho score and the MAI hostility score and the MAI, and the least association with the MAI anger-in score and the JAS (Table A3.7). Similar strong associations were observed between these scores in the young control group without CHD (Table A3.8)

A3.1.7.3 Multivariate regression analysis. In the young CHD group, multivariate analysis with coronary artery scores as the dependent variables, and parameters having a linear association (Ho, PACKYR, BMI, DBP, CHOL, ETOH and HbA1C) as the independent variables, demonstrated no independent association for the MMPI Ho score. Similarly in the older CHD group, the MMPI Ho score did not maintain an independent predictive relationship with any of the measures of the coronary artery scores. Thus, the predictive utility of the MMPI Ho score was not sustained in multivariate analysis in this study.

A3.1.8 Discussion

Conclusively demonstrating a consistent, much less causal relationship between psychosocial variables, behavioural factors and CHD has proved to be very difficult. A meta-analysis of specific variables has indicated a small but reliable association between Type A behaviour, depression, anger, hostility, aggression and anxiety, and CHD.¹¹¹⁵ Nevertheless, a great deal of concern exists about the validity of a number of these factors, including hostility (as defined by the MMPI Ho score) being risk factors for CHD.^{1141,1154,1156,1174} Unfortunately, the available methods to assess hostility are influenced by the individual's test-taking attitude, which can bias the MMPI profiles.¹¹⁷⁵ Also, there is serious concern about what can be expected in normal subjects using the MMPI Ho score.¹¹⁷⁵ Furthermore, the exact nature of what the MMPI Ho score is measuring probably needs further refinement.¹¹⁵⁸

Nevertheless, there is still highly suggestive data to implicate hostility as a risk factor for CHD, and it is clear that further information about the relationship between CHD, hostility and other psychosocial variables is necessary. In this regard, in the present study the differences in the measures of hostility in young males with and without CHD, the relationship of the MMPI Ho score to angiographically defined CAD, the evaluation of alternative measures of hostility and anger, and the interaction of the hostility measures with other risk factor for CHD add further unique knowledge for consideration.

A3.1.8.1 Type A Behaviour. The results are consistent with the current body of literature indicating that the type A behaviour, measured as a global construct, may not be a good predictor of CHD, and in fact may not be a risk factor for CHD at all. Similar negative findings were observed in the majority of angiographic studies,^{209,985,1112} whether using the SI, the JAS or the Bortner scale.⁹⁸⁵ However, the results do need to be interpreted cautiously because the JAS is an inferior measure of type A behaviour compared to the SI.³⁷⁷ The JAS was chosen because of the inherent problems associated with the SI, in particular concern regarding the validity of results obtained by less experienced interviewers.¹¹⁶⁹

A3.1.8.2 Anger. Progress in the understanding of the role of anger in the aetiology of CHD requires conceptual clarification of the components of the attitudes within what is called anger, and the construction of objective, reliable and valid measures of these components.¹³² However, the purpose of using the measures of anger (MAI) and an alternative assessment of hostility (MAI Ho score) in this study was not to undertake a comparison or factorial analysis of the MMPI Ho score and anger, but to evaluate their comparative relationship to CHD and risk factors for CHD in well

defined study groups. Furthermore, it is imperative to determine if alternative measures of hostility can substantiate the association previously documented between the MMPI Ho score and CHD.

Anger and aggression have long been regarded as important factors in CHD. These personal characteristics may also be confounding factors in the relationship between hostility and CHD. In addition, since the MMPI Ho score may not be measuring hostility *per se*, but other attitudes, behaviours and/or traits, such as cynicism and mistrust,¹¹⁵⁸ the need to validate hostility as a risk factor for CHD is essential. If such a relationship is confirmed, attention may then be directed to develop methods of intervention. Moreover, when using multiple comparisons to assess relationships between variables, the validity of a significant association is enhanced by demonstrating reproducible unique correlations.

The results demonstrate a clear lack of correlation between CHD and anger, as measured by the MAI, as well as suppressed anger measured by this inventory. Equally important, the construct in the MAI measuring "hostile-outlook" also had no consistent relation to CHD and did not differentiate between cases and controls without CHD.

A general measurement of anger, suppressed anger or a hostile-outlook component of a general anger construct are not associated with CHD. The methods used in this study, however, did not apply a state-trait distinction for anger or hostility. Although the MMPI Ho score does have adequate stability over time as reflected in a high ($r=0.86$) test-retest correlation,^{1157,1171} uncertainty exists as to whether the MMPI Ho score reflects a persisting psychological trait in an individual or whether it is influenced significantly by circumstances at the time of the measurement and which do not necessarily reflect a long-term characteristic. Nevertheless, research on hostility (and anger) needs to further clarify whether such constructs refer to transitory emotional states and/or relatively stable individual differences in personality traits.¹¹⁵⁷

A3.1.8.3 Hostility. The consistent association in this study between the MMPI Ho score and angiographically defined CAD, in particular, the severity of CAD, supports the findings of previous studies in different population groups using different methods of assessing hostility.^{1152,1153} The association with severity also supports those prospective studies demonstrating an association with acute cardiac events,^{1118,1147,1151} events which are often related to the severity of the underlying CAD. Moreover, in persons with established CHD, extreme hostility appears to impair the survival of patients with CHD.¹¹²⁰ Such an observation is consistent with an association, whether causal or secondary, between hostility and severity of CAD defined angiographically. The relationship of the MMPI Ho score to CAD or CHD is, however, controversial,¹¹⁴¹ and the results from this study differ from some published observations.^{1141,1154,1156,1174} Others have found that the relationship is only present in subgroups, such as hypertensive men.¹¹²⁰

Unfortunately many factors exist which can confound results of studies utilising angiographic methods to assess the significance of risk factors for CHD.⁹⁸⁵ The cholesterol level has been the one consistent risk factor to be associated with angiographically defined CAD in a number of studies, and the presence of a similar rela-

tionship can be considered supportive of the validity of the angiographic measures used.⁹⁸⁵ In one study with differing results to the present study, the cholesterol level and age of the study group were not related to CAD in one study¹¹²³ and not evaluated in the other,¹¹⁴¹ contrary to the current study. This lack of association places in doubt the validity of the CAD scoring methods used in the two reports. In one study, patients who had had a myocardial infarct more than 6 months before the study were excluded.¹¹²³ This could result in a selection bias, by reducing the number of patients with more severe disease. Indeed, the fact that the mean occlusion score was skewed to the right would support such an interpretation.

Our findings demonstrated the strongest association between the MMPI Ho score and disease severity. An important fact in a previous study¹¹²³ is that the questionnaire was designed to obtain information on risk behaviour prior to the onset of symptoms. In the present study there was an association between the MMPI Ho score and the NYHA functional class for angina in the older age CHD group. Therefore, our study may be biased by the potential of symptoms to cause increased hostility in the group, with the more severe the disease the more the symptoms. Although prospective studies could overcome such a bias, those to date have been conflicting, some supporting an association between hostility and CHD,^{1118,1147,1151} and others not.^{1144,1154-1156}

In the present study, there was no association between the MAI hostility score, which gives a measure of hostile-outlook, that is, a state rather than a trait measurement, and CHD or functional capacity. Furthermore, measures of extreme hostility do not appear to be a consequence of symptom severity.¹¹²⁰

A3.1.8.4 Risk factors for CHD and Hostility. Using a different measure of hostility, hypertension, heavy smoking and heavy use of alcohol have been documented to be associated with extreme hostility, but not with obesity.¹¹²⁰ In our study, there was an association in the younger, but not in the older aged CHD group, between hostility and both alcohol intake and previous smoking. The potential for an interaction between environmentally related risk factors and behavioural characteristics is not surprising. Such a relationship provides a source for the link between differing behavioural characteristics and CHD. Although, in the analysis by multiple regression, the MMPI Ho score was not independently predictive of CAD and therefore may not be a casual risk factor for CHD, this may equally indicate that the measure simply has a lower order of risk compared to the traditional major risk factors, particularly smoking, hypertension and cholesterol levels.

A3.1.8.4 Limitations of an angiographic study. The methods of assessing coronary angiograms vary greatly, and differences in results between studies may clearly relate to such methodological differences.⁹⁸⁵ To attempt to limit this problem multiple scoring methods were used and the results were reasonably consistent. Bias can arise from the selection of subjects, particularly if there is little variation in the measure applied. In this study, CHD cases were compared to a normal control group. Furthermore, the patients evaluated did have a demonstrable relationship with other stronger and more accepted risk factors, in particular the cholesterol level. The validity of a negative finding is thus supported by the relationship.

A type II statistical error may very well have lead to any negative findings, missing a weak relationship because of an inadequate sample size. However, predictable differences and associations of the classic risk factors were able to be detected. That is, clinically meaningful and established differences and associations were determined from the sample number used in the study. Weaker and less clinically relevant relationships may have been missed in this study. Even though a predictive relationship between the CAD score and other major risk factors can be demonstrated, indicating that any potential relationship between CAD and a measure of hostility is less predictive than those other variables entered into the regression analysis, the results do not entirely exclude a weaker relationship.

There may be an effect of medications on the psychosocial and behavioural measures, especially beta-blockers. However, this may only be relevant for the SI and not for an administered self-report questionnaire such as the JAS.⁹⁸⁵

Anatomic CAD may not be the right end point for the expression of some CHD risk factors,⁹⁸⁵ as there is a high probability that there are risk factors for various stages in the development of the disease.²⁰²

The results of this study support the need for ongoing research into the relationships between psychosocial and behavioural factors, and CHD. Further information is also required about the interrelations of the different psychological, psychosocial, behavioural and personality predictors of CHD.¹¹¹⁵

An important outcome of research on the psychological predictors of heart disease in more recent times, has been to dispel the image of the coronary-prone individual as being a hurried, impatient workaholic.¹¹¹⁵ Indeed, contemporary information would indicate that the "coronary prone" individuals are more likely to be those with one or more negative emotions.¹¹¹⁵

A3.1.9 Summary

1. In the two groups of CHD patients evaluated in this study, a reproducible univariate correlation between the MMPI Ho score and CAD has been shown.

2. The relationship is not sufficiently strong, in comparison to other risk factors or because of an association with the CHD risk factors, to remain independently predictive of CAD following multivariate analysis.

3. In the case-control study, at least one standard behavioural measure (the MMPI Ho score) may be an independent CHD risk factor which was not controlled for in the case-control study design. This measure was potentially a confounding factor in the assessment of platelet function as a risk factor.

A3.2 HOSTILITY AND PLATELET FUNCTION IN PATIENTS WITH CORONARY HEART DISEASE

A3.2.1 Introduction

There exists evidence which is suggestive that mental stress may increase *in vivo* and *in vitro* platelet reactivity.^{1174,1176} Indeed, stress and other sympathoadrenal activation seems to activate or sensitise platelets as measured by a number of different methods.¹¹⁷⁶ An understanding of the biological health consequences of hostility, or other similar characteristics, is presently undeveloped.³⁷⁸ Excessive cognitively mediated neuroendocrine and cardiovascular responses, and an inadequate parasympathetic antagonism of sympathomimetically mediated responses occur in hostile type A persons.³⁷⁸ Although large elevations of plasma adrenaline produced by infusions or hypoglycaemia enhance *in vivo* platelet function, the effects of any changes associated with psychosocial conditions potentially producing increased neuroendocrine responses is unclear.¹¹⁷⁶ Indeed, even though the biological mechanism(s), if any, responsible for the adverse health consequences of the hostile trait, state or hostility complex is not established.^{376,378} Nevertheless, one plausible mechanism relates to the potential influence of altered platelet function.³⁷⁶

In light of the group differences in the MMPI Ho score between the young control group and the young CHD group, and the relationship of the MMPI Ho score with angiographically defined CAD, this section evaluated the relationship between measures of hostility and platelet function.

A3.2.2 Methods

The methods and patient population are described in section A3.1.

A3.2.3 Results

The control group did not have a significant relationship between any measure of platelet function and the psychological scores apart from the MMPI Ho score with LT50ADR ($r=-0.380$, $p=0.033$), anger-in (MAI) with LT50ADP ($r=-0.351$, $p=0.01$), the JAS with RCOL ($r=-0.305$, $p=0.014$), the hostility score of the MAI with LT50ADP ($r=-0.340$, $p=0.012$) and the MAI with LT50ADP ($r=-0.341$, $p=0.030$). These measures did not have other significant associations.

The young CHD group had correlations between the MMPI Ho score and the LT50ADR, the RADR and RADP (Table A3.9). There were significant correlations between MAI and RADP, and JAS with LT50COL (Table A3.9).

A3.2.4 Discussion

A consistent positive relationship between platelet aggregation measures and the MMPI Ho score in the young CHD group has been demonstrated. Only isolated associations were noted with other psychological measures and no consistency of association was noted in the control group. In other words, these associations found probably arise from multiple comparisons. Given the association with the MMPI Ho score and adrenaline induced platelet aggregation in particular, the MMPI Ho score will need to be controlled for in the final comparison analysis and multivariate analysis with angiographically defined CAD.

Table A3.1. Values for Behavioural Psychological Measures.

	Young Controls	Young CHD Group	Older CHD Group
Ho (MMPI)	15+/- 6	21.2+/- 8.5	23.3+/- 8.7*
JAS	198+/-83	265 +/-80	248 +/-82
MAI	100+/-17	113 +/-20	108 +/-25
Ho (MAI)	28+/- 6	32 +/- 7	31 +/- 8
Anger	17+/- 5	19 +/- 5	18 +/- 6

* ANOVA F=16.82, uncorrected p<0.0001

Young controls versus young CHD group:

mean difference = 6.2, Bonferroni p<0.001

Young controls versus older CHD group

mean difference = 8.3, Bonferroni p<0.001

Ho (MMPI)=hostility score from the Minnesota Multiphase Personality Inventory, JAS=Jenkins activity survey, MAI=multidimensional anger inventory, Ho (MAI)=hostility score from the MAI, Anger=the anger-in score from the MAI.

Table A3.2. Linear correlations with CHD risk factors in the younger CHD group.

	Ho(MMPI)	Ho(MAI)	MAI	Anger(MAI)	JAS
DOB	-0.219	-0.058	0.018	-0.194	-0.026
FC	0.069	0.050	0.149	-0.084	0.048
AMI	0.022	0.049	-0.102	-0.020	0.254
PACKYR	0.486#	0.176	0.083	0.219	0.404*
ETOH mg	0.329*	0.229	0.352*	0.192	0.261
DBP	0.313*	0.335*	0.330*	0.152	0.024
SBP	0.187	0.253	0.251	0.143	-0.143
BMI	0.340*	0.209	0.251	0.148	-0.003
WT	0.127	0.105	0.222	0.280	-0.049
CHOL	0.338*	0.157	0.262	0.378*	0.016
TRIG	-0.134	-0.019	-0.026	0.144	-0.259
HDL	0.374*	0.031	0.019	-0.145	0.248
LDL	0.285	0.165	0.284	0.433**	-0.016
APOA1	0.552^	0.389*	0.353*	0.068	0.410**
APOB	0.292	0.030	0.159	0.300*	-0.009
FIBRIN	0.266	0.229	0.118	0.059	0.033
BSL	0.229	0.404**	0.310*	0.121	-0.203
HbA1C	0.336*	0.313*	0.264	0.140	-0.151

*p<0.05, **p<0.02, #p<0.005, ##p<0.000

Abbreviations are explained in Tables 6.1 and A3.1.

Table A3.3. Linear correlation between psychological measures and angiographic scores in the younger CHD group.

	Ho(MMPI)	Ho(MAI)	MAI	Anger(MAI)	JAS
Normseg	-0.308 [^]	-0.160	-0.095	-0.142	-0.245
>50%	0.065	-0.206	-0.236	-0.045	-0.090
>70%	0.190	0.037	0.027	-0.028	0.233
CSS	0.349*	0.291	0.239	0.071	0.043
mCSS	0.353*	0.274	0.232	0.054	0.048
CAS	0.232	0.208	0.174	0.131	-0.033
mCAS	0.269	0.192	0.173	0.130	0.007
Jenkins	0.247	0.134	0.138	0.083	-0.009
LVS	0.077	0.124	0.008	-0.006	-0.169
CS	0.297*	0.314*	0.318*	0.183	-0.136

*p<0.05, #p<0.02, ^p<0.005

Abbreviations are explained in Tables 6.3 and A3.1.

Table A3.4. Linear correlation with other behavioural measures in the younger CHD group.

	Ho(MMPI)	Ho(MAI)	MAI	Anger(MAI)	JAS
Ho	-	0.669##	0.626##	0.350*	0.334*
Ho (MAI)	0.669##	-	0.840##	0.563##	-0.046
MAI	0.626##	0.840##	-	0.777##	-0.029
ANGER-IN	0.350*	0.563##	0.776##	-	-0.185
JAS	0.334*	-0.046	-0.029	-0.185	-

*p<0.05, **p<0.02, #p<0.005, ##p<0.000

Abbreviations are explained in Table A3.1.

Table A3.5. Linear correlation with CHD risk factors in the older CHD group.

	Ho(MMPI)	Ho(MAI)	MAI	Anger(MAI)	JAST
DOB	-0.249#	0.186	0.244*	0.293#	0.136
FC	0.242#	-0.013	-0.016	0.029	-0.028
AMI	0.140	0.134	0.031	0.048	0.210*
PACKYR	0.001	0.111	0.163	0.040	0.128
ETOH mg	0.082	0.043	0.206*	0.219*	0.042
DBP	-0.129		0.135	-0.066	0.110
SBP	0.142	0.236	0.267	0.159	-0.058
BMI	0.317*	0.226	0.031	-0.122	-0.142
WT	0.104	0.115	0.192	-0.264	-0.012
CHOL	0.226*	-0.042	0.129	0.230*	0.133
TRIG	-0.144	-0.071	-0.044	0.006	0.003
HDL	0.174	-0.129	0.029	-0.026	0.040
FIBRIN	0.107	-0.084	-0.304*	-0.172	-0.165
BSL	0.186	0.218	0.198	0.186	-0.201

*p<0.05, **p<0.02, #p<0.005, ###p<0.000

Abbreviations are explained in Tables 6.1 and A3.1.

Table A3.6. Linear correlation with angiographic scores in the older CHD group.

	Ho(MMPI)	Ho(MAI)	MAI	Anger(MAI)	JAST
Normseg	-0.300^	0.017	0.193*	0.222*	-0.102
>50%	0.217*	-0.239*	-0.302**	-0.166	-0.021
>70%	0.248#	-0.104	-0.117	-0.065	0.061
CSS	0.395^	-0.026	-0.146	-0.142	-0.007
mCSS	0.363^	-0.070	-0.166	-0.173	0.002
CAS	0.235*	-0.016	-0.138	-0.149	0.048
mCAS	0.240*	-0.054	-0.159	-0.179	0.059
Jenkins	0.321^	-0.037	-0.106	-0.106	0.066
LVS	0.085	-0.005	-0.088	-0.078	-0.086
CS	0.233*	0.122	0.004	-0.017	-0.096

*p<0.05, **p<0.02, #p<0.005

Abbreviations are explained in Tables 6.3 and A3.1.

Table A3.7. Linear correlation with other behavioural measures in the older CHD group.

	Ho(MMPI)	Ho(MAI)	MAI	Anger(MAI)	JAS
Ho	-	0.467##	0.435##	0.302#	0.318#
Ho (MAI)	0.467##	-	0.800##	0.622##	-0.336#
MAI	0.489##	0.800##	-	0.829##	-0.408
ANGER-IN	0.388#	0.622##	0.829##	-	-0.264*
JAS	0.314*	0.336#	0.408##	0.264	-

*p<0.05, **p<0.02, #p<0.005, ##p<0.000

Abbreviations are explained in Table A3.1.

Table A3.8. Linear correlation with other behavioural measures in the young control group.

	Ho(MMPI)	Ho(MAI)	MAI	Anger(MAI)	JAS
Ho	-	0.769#	0.689#	0.547**	0.191
Ho (MAI)	0.770#	-	0.881##	0.748#	0.202
MAI	0.689#	0.881##	-	0.914##	0.157
ANGER-IN	0.547**	0.563##	0.913##	-	-0.006
JAS	0.191	0.202	0.157	-0.005	-

*p<0.05, **p<0.02, #p<0.005, ##p<0.000

Abbreviations are explained in Table A3.1.

Table A3.9. Linear correlation with platelet function in the young CHD group.

	Ho(MMPI)	Ho(MAI)	MAI	Anger(MAI)	JAST
LT50ADR	-0.518#	-0.155	-0.228	-0.254	-0.029
RADR	0.441#	0.138	0.189	0.235	0.029
LT50ADP	-0.201	-0.070	-0.029	-0.023	-0.050
RADP	0.433#	0.251	0.350*	0.277	0.191
LT50COL	-0.280	0.007	-0.119	-0.185	-0.310*
RCOL	0.156	-0.066	0.072	0.021	0.226
LAG	-0.247	0.020	0.022	-0.159	-0.073
TXB2	-0.106	0.010	-0.210	-0.223	-0.119
BTG	0.176	0.226	0.225	0.074	-0.085
MPV	-0.072	-0.071	-0.082	0.017	0.275
PCR	-0.011	-0.131	-0.119	-0.170	0.131

Abbreviations are explained in Table 6.1, 6.2 and A3.1.

Table A3.10. Regression analysis with coronary artery scores as dependent variables, and parameters with a univariate relation (Ho, ETOH, PACKYR, BMI, DBP, CHOL, APOA, RADP, and LT50ADR) as independent variables for the young CHD group.

Dependent Variable	Independent Variable	B	SE B	Beta	R sq	P value
CSS	LT50ADR	-25.58	8.460	-0.700	0.504	0.014
mCSS	LT50ADR	-27.70	7.824	-0.763	0.582	0.006
CAS	Nil					
mCAS	Nil					
NORMSEG	APOA	-0.079	0.034	-0.608	0.370	0.047

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